AD_____

Award Number: DAMD17-98-1-8031

TITLE: Metal Ion Complexes for Antibody Imaging and Therapy in Breast Cancer

PRINCIPAL INVESTIGATOR: John E. Shively, Ph.D.

CONTRACTING ORGANIZATION: Beckman Research Institute of the City of Hope
Duarte, California 91010

REPORT DATE: March 2002

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

20020827 045

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	March 2002	Final (1 Aug 98 - 28 Feb 02)

4. TITLE AND SUBTITLE
Metal Ion Complexes for Antibody Imaging and Therapy in Breast Cancer

5. FUNDING NUMBERS
DAMD17-98-1-8031

6. AUTHOR(S)
John E. Shively, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Beckman Research Institute of the City of Hope
Duarte, California 91010

E-Mail: jshively@coh.org

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
report contains color

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*
The goals of this project were to prepare and test novel bifunctional chelates based on DOTA to anti-CEA and anti-her2/neu antibodies. The conjugates were labeled with In-111 for imaging and Y-90 for therapy. Two tumor xenograft models were tested: MCF7 cell transfected with her2/neu for over-expression of her2/neu, and MCF7 cell expressing "normal" amounts of her2/neu. Both cell lines also express CEA. We showed that both models had good uptake of In-111 labeled antibodies and responded to Y-90 labeled RIT. The RIT response was further improved by the combined administration of taxol and RIT. Novel peptide-DOTA conjugates to recombinant antibody fragments were prepared, radiolabeled with In-111, and shown to reduce normal kidney uptake compared to DOTA directly conjugated to the same antibody fragments. This work demonstrates that breast cancers can be treated with both anti-her2/neu and anti-CEA antibodies, and that recombinant antibody fragments can be used for imaging or therapy with the novel peptide linker chelators.

14. SUBJECT TERMS
her2/neu, CEA, chelate, antibody, radioimaging, radioimmunotherapy, breast cancer

15. NUMBER OF PAGES
29

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

FINAL REPORT

Reporting period: 8/1/98 – 2/28/02

1.0 Introduction

Breast cancer can be targeted with radiolabeled anti-tumor antibodies. In this project we chose anti-CEA antibody cT84.66 for targeting CEA positive breast cancers (about 50% are CEA positive) and anti-Her2/neu antibody 4D5 for Her2/neu positive breast cancer (about 30% are Her2/neu positive). Both antibodies, cT84.66 (chimeric) and humanized 4D5 (Herceptin), are well characterized and have been used clinically. The novel aspects of this project are the use of novel chelates to improve the biodistributions and tumor to blood ratios of the radiolabeled antibodies. The radioisotopes are ^{111}In (2.8 day half life, pure gamma emitter) for imaging and ^{90}Y (64h half life, pure beta emitter) for therapy. This is a preclinical study to determine the optimum chelate for each antibody. The original animal model was a CEA positive/Her2/neu positive cell line grown as a xenograft in nude mice. Since work over the first year suggested that this cell line was sensitive to cold 4D5 in vivo, we also explored use of the parent cell line MCF7, which is also CEA and Her2/neu positive. In addition, we began using the humanized version of 4D5, Herceptin.

2.0 Body

Cell lines. The MCF7/Her2/neu cell line was obtained from Dr. Dennis Slamon at UCLA. The cell line is positive for both CEA and Her2/neu which was transfected into the parent line MCF7 (1). These cells were analyzed for Her2/neu by FACS using the 4D5 antibody. The results (**Figure 1B**) show intense staining for Her2/neu compared to the parent cell line (**Figure 1D**).

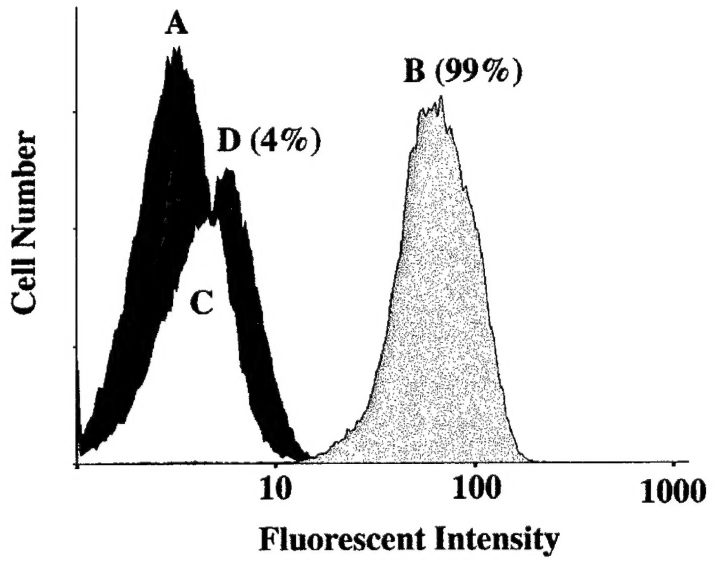


Figure 1. FACS analysis of MCF7/Her2/neu and MCF7 cells with anti-Her2/neu antibody 4D5. **A.** MCF7/Her2/neu cells, secondary antibody only. **B.** MCF7/Her2/neu cells stained with 4D5 (99% positive). **C.** Parental line MCF7, secondary antibody only. **D.** Parental line MCF7 stained with 4D5 (4 % positive).

Antibody conjugates. 4D5 or Herceptin was conjugated to DOTA (1,4,7,10-tetraazacyclododecane- N,N',N'',N'''tetraacetic acid) using our previously published active ester method (2). Briefly, the antibody (2 mg in 1 mL of PBS) was mixed with EDC (1-ethyl-3-[3-dimethylamino)propyl] carbodiimide) and sulfo-N-hydroxysuccinimide at a ratio of 1:100 for 1 h at room temperature and then dialyzed into 0.2 M ammonium acetate pH 5 buffer. The DOTA conjugated antibody was radiolabeled with either ^{111}In or ^{90}Y in the ammonium acetate buffer for 1 h at 43°C. The radiolabeled antibody was separated from free isotope after the addition of 10 mM DTPA (diethyltriaminopentaacetic acid) by gel filtration chromatography (TosoHaas TSK G2000, 10 um, 7.5 x 300 mm) in normal saline at a flow rate of 0.5 mL/min and monitored by A280 nm and radioactivity. Based on this analysis, incorporation of radioisotope was 80%. The number of chelates per antibody (5.0) was determined by using radiotracer tagged $^{111}InCl_3$. The immunoreactivity of the radiolabeled antibody was shown to be 95% based on a cell binding assay. Based on these analyses, we conclude that 4D5 or Herceptin can be conjugated to DOTA without loss of immunoreactivity and can be efficiently radiolabeled with either ^{111}In or ^{90}Y.

Animal biodistributions. Animal bidistributions for ^{111}In-DOTA-4D5 using the MCF7/Her2/neu cells in nude mice have been published by us (4). New studies using the MCF7 parental line and ^{111}In-DOTA-Herceptin have been completed. Groups of five mice per time point bearing MCF7 xenografts were injected with 5.0 uCi of ^{111}In-labeled DOTA-Herceptin (2.7 ug antibody per mouse) via tail vein. Animals were sacrificed at 0, 5, 24, 48, 72 and 96 hours post injection. Tumors were dissected and the major organs and blood weighed and the activity measured. The activity expressed in percentage injected dose per gram of tissue was then calculated. The mean values were used to construct biodistribution curves for tumors and normal organs. The results are shown in **Table 1** and **Figure 2**. Tumor uptake for the low Her2/neu expressing cell line (MCF7) is almost equivalent to the her2/neu over-expressing line, suggesting that both types of patients would qualify for treatment.

Table 1. Biosdistribution of In^{111} labeled DOTA-conjugated Herceptin in nude mice bearing MCF7 xenografts.

Organ	Time (h)			
	0 &	24	48	72
Blood	40.91 (3.76)	15.83 (2.80)	11.96 (2.43)	10.84 (3.01)
Liver	7.60 (0.48)	5.05 (0.58)	5.39 (1.54)	6.90 (1.45)
Spleen	5.59 (1.00)	3.76 (1.04)	3.31 (0.67)	3.15 (0.82)
Kidney	6.62 (0.78)	4.80 (0.73)	4.17 (0.70)	4.10 (0.29)
Lung	11.16 (2.09)	6.77 (2.31)	4.92 (1.15)	4.28 (0.89)
Bone	1.84 (0.48)	1.78 (0.34)	1.52 (0.52)	1.31 (0.21)
Tumor	0.95 (0.22)	16.91 (2.64)	25.4 (5.60)	24.94 (3.31)
Carcass	1.54 (0.27)	2.71 (0.44)	2.33 (0.38)	2.24 (0.36)

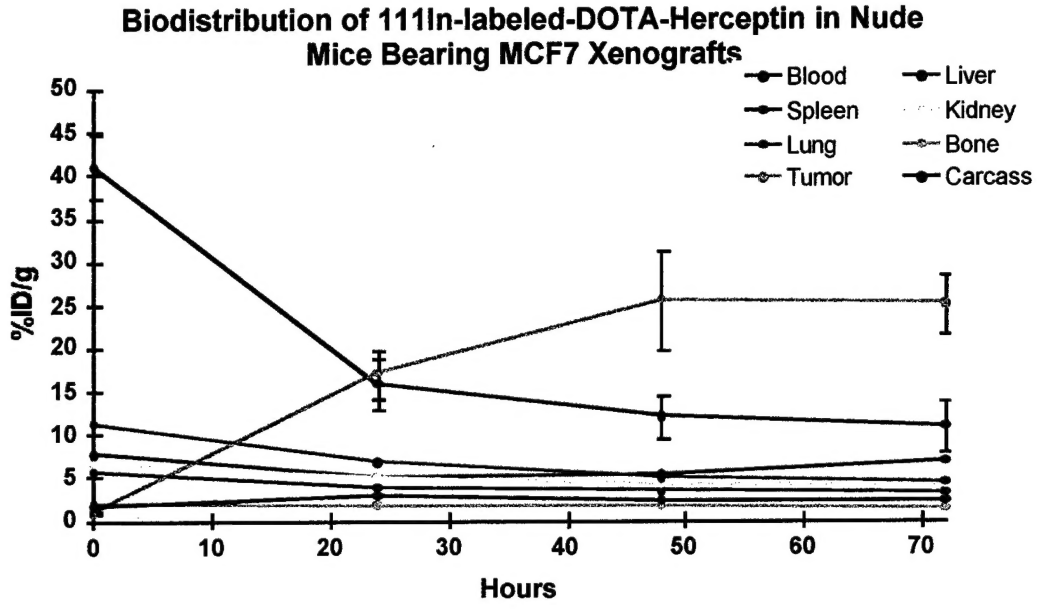


Figure 2. Biosdistribution of In^{111} labeled DOTA-conjugated Herceptin in nude mice bearing MCF7 xenografts.

Radioimmunotherapy with ^{90}Y-herceptin in the animal model. Using the MCF7/her2/neu model, 50 or 100 uCi of ^{90}Y labeled DOTA-4D5 or Herceptin was administered to groups of 9 animals. Control groups were injected with 100 uCi of ^{90}Y labeled DOTA-Leu16 (irrelevant antibody), unlabeled DOTA-4D5 (3 ug, the same amount of antibody as in the radiolabeled group) or saline. Tumor volumes (L x W^2/2)were measured twice weekly and relative tumor volumes calculated (compared to RIT at day 1. The results with ^{90}Y labeled DOTA-4D5 (4, **Figure 3**) show that mice injected with 50 uCi of ^{90}Y-labeled DOTA-4D5 had a two-fold reduction in tumor growth compared to control groups at the end of 37d ($P < .01$, ANNOVA). For those treated with 100 uCi of radiolabeled antibody, tumor growth was reduced 2.7 fold($P < .001$, ANOVA). Since this was only a single injection, tumor regrowth started to occur at 19d. There were no tumor cures with the single injection of ^{90}Y labeled DOTA-4D5.

The results with ^{90}Y labeled 1B4M-Herceptin plus or minus taxol in the MCF7 model are shown in **Figure 4**. It can be seen that RIT with either 40 or 80 uCi gave significant tumor growth reduction ($P<.001$, ANOVA). Thus, the MCF7 cels that express "normal" amounts of her2/neu re more sensitive to RIT than yjose transfected with her2/neu and over-expressing the antigen (compare **Figure 3** vs **Figure 4**). In addition, we tested the effect of adding taxol to the RIT. Tumor growth retardation with the addition of two doses of taxol (300 ug, IP) was even greater, leading to complete absence of tumors in some animals ($P <.001$, ANOVA). Thus, we hypothesize that a clinical trial with herceptin RIT plus taxol would be more effective than RIT alone and would perform best in patients with "normal" levels of her2/neu. This is an important observation, since only those over-expressing her2/neu respond to cold herceptin.

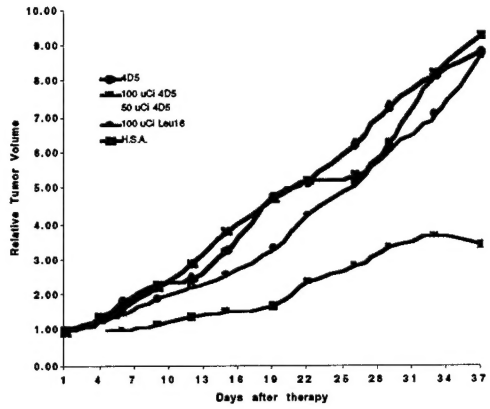


Figure 3. RIT with ^{90}Y-herceptin in nude mice bearing MCF7/her 2neu xenografts . Groups of nine mice were treated with with cold 4D5 (3 ug), HSA (no mAb control), 4D5 (3 ug) labeled with either 50 or 100 uci of ^{90}Y DOTA-4D5 or 100 uCi ^{90}Y DOTA-Leu6 (irrelevant mAb control).

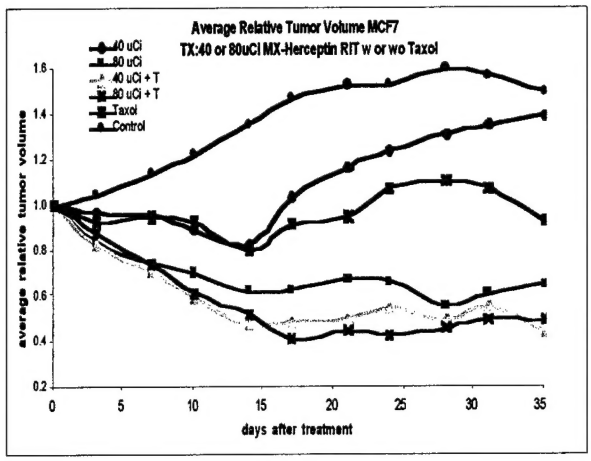


Figure 4. RIT with ^{90}Y-herceptin plus or minus taxol in nude mice bearing MCF7 xenografts. Brown: untreated controls. Blue: 40 uci RIT. Purple: 300 ug taxol IP, 2x. Magenta: 80 uCi RIT. Orange: 40 uCi RIT plus taxol (300 ug, 2X). Green: 80 uCi RIT plus taxol (300 ug, 2x).

Radioimmunotherapy with ^{90}Y-cT84.66 in the animal model. Since many breast cancers express CEA, including the MCF7 cells used in our animal model, we tested their response to the ^{90}Y- labeled anti-CEA antibody cT84.66. Although we had previously shown that ^{111}In-labeled cT84.66 localizes to MCF7 tumor xenografts, a biodistribution study was performed to demonstrate that uptake in the xenograft was significant. The results (not shown) demonstrated a very low but significant uptake (about 15%ID/g at 48h). The reason for the low uptake compared to radiolabeled herceptin is unknown, but it is noteworthy that the cells are CEA positive when analyzed by the same antibody in FACS (data not shown). Nonetheless, we proceeded to perform an RIT study to determine if the uptake was sufficient for a therapeutic effect. The results are shown in **Figure 5.** In this experiment both 50 uCi and 100 uCi of RIT produced a significant reduction in tumor size (P<.001, ANOVA), demonstrating comparable results to similar activities of RIT with ^{90}Y-labeled herceptin in the same animal model (**Figure 4**). In addition, we combined anti-CEA RIT with cold herceptin. In this model, we had previously shown that doses of cold herceptin in the range of 100 ug performed twice (0 and 9d, IP) was sufficient to cause tumor growth reduction without actually killing the tumors (**Figure 5**, purple curve). When this dose regimen was combined with either 50 or 100 uCi of anti-CEA RIT, there was no further improvement in the effects of RIT alone. In order to determine if there is an additive or synergistic effect, we need to repeat this experiment with even lower doses of RIT.

The combination of anti-CEA RIT and taxol was also tested. In this experiment we lowered the amount of RIT to permit a better analysis of possible additive or synergistic effects. Both doses of RIT caused mild reduction of tumor size, while taxol only had a more significant reduction of tumor size compared to untreated controls (P<.001, ANOVA). However, the combied therapy had an additive effect (P<.001, ANOVA0, demonstrating that RIT with either anti-her2/neu or anti-CEA combined with taxol are viable options for the clinic.

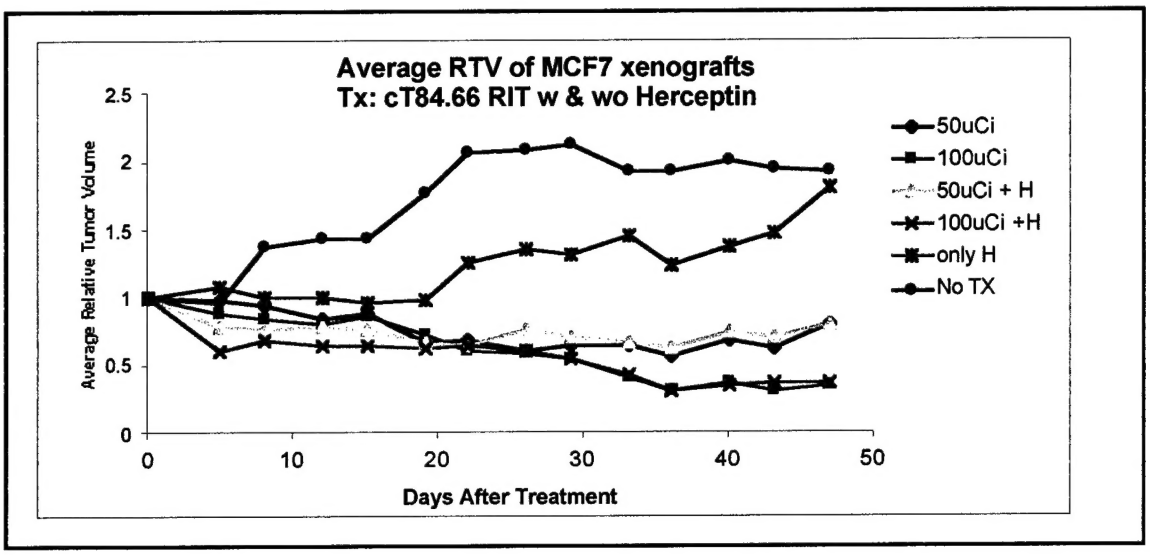


Figure 5. RIT of MCF7 xenografts in nude mice with ^{90}Y-DOTA-cT84.66 plus or minus cold herceptin. Groups of seven mice beraing MCF7 xenografts were treated with 50 or 100 uCi of RIT plus or minus treatments with cold herceptin. Red: no treatment. Purple: cold herceptin only (100 ug, 2x). Blue: 50 uCi RIT only. Magenta: 100 uCi RIT only. Orange: 50 uCi RIT plus cold herceptin. Green: 100 uCi RIT plus cold herceptin.

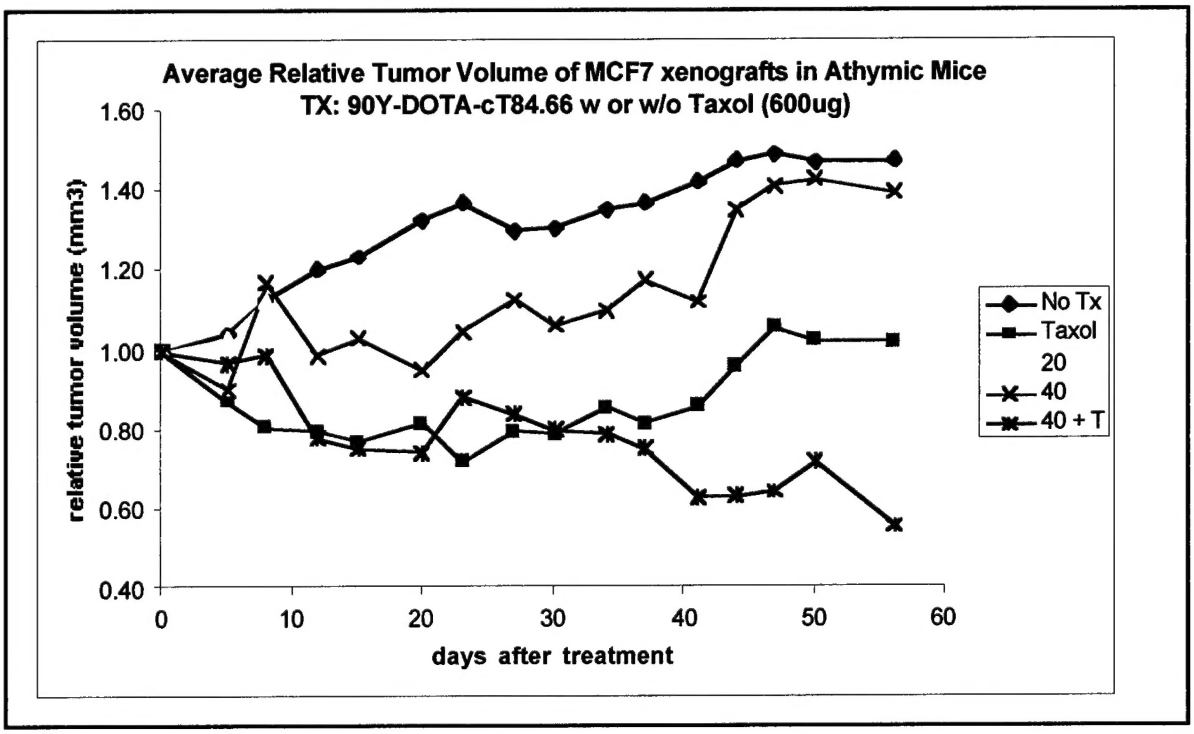


Figure 6. RIT of MCF7 xenografts in nude mice with ^{90}Y-DOTA-cT84.66 plus or minus taxol. Groups of 7 mice bearing MCF7 xenografts were treated with either 20 or 40 uCi of anti-CEA RIT plus or minus taxol. Dark blue: No treatment. Yellow: RIT with 20 uCi. Light blue: RIT with 40 uCi. Magenta: taxol only (300 ug, 2x, IP). Purple: 40 uCi RIT plus taxol.

Evaluation of new chelates. In the above work, DOTA conjugated to intact antibodies was evaluated. The simplified method for conjugation of the active ester of DOTA to antibodies was developed by us (2). We also developed a method that inserts a chemically labile linker between the antibody and DOTA (MC-DOTA; 5). In the SOW we had proposed to (a) test the

effect of linker length on the conjugation and radiolabeling efficiency and to test the best derivatives in an animal model; (b) to oxidize the thioether linkage to a sulfone or a sulfoxide and repeat the tests; (c) to conjugate the sulfhydryl specific reagents to Fab' fragments and repeat the tests; and (d) to synthesize a novel DOTA chelator (DOTA-allyl amide). The progress on each of these goals is addressed below.

(a) Effect of linker length on conjugation efficiency. MC-DOTA has a hexane (C6) bis-maleimide linker. Butane (C4) and ethane (C2) bis-maleimide linkers are commercially available. Each was coupled to Cys-DOTA generating the following reference bifunctional chelators: MC-C6-DOTA, MC-C4-DOTA, and MC-C2-DOTA. Each was purified by RP-HPLC and shown to have a single peak and the correct mass by ESI-MS. Each compound was conjugated to mildly reduced cT84.66 and a test radiolabeling with In-111 was performed. MC-C6-DOTA, the reference standard (5) was radiolabeled with In-111 with an efficiency of 82% (10 mCi/mg), MC-C2-DOTA was labeled with 78% efficiency, and MC-C4-DOTA with an efficiency of <2%. Since the results with the MC-C4-DOTA were unexpected, the conjugation was repeated twice and shown to be reproducibly low. Since we had no theoretical reason for this result, the three compounds were modeled to determine the possibility of the formation of an unreactive species. Molecular modeling did not provide any insights into the nature of the problem. Stability studies of MC-C2-DOTA demonstrated that this compound had comparable stability to MC-C6-DOTA. Since the stabilities were comparable, and MC-C6-DOTA conjugated to cT84.66 hab been extensively studied (6), no further studies were performed on MC-C4-DOTA-T84.66.

(b) Effect of oxidation of thioether linkage to sulfone or sulfoxide. Oxidation of the thioether to either the sulfone or sulfoxide increases the pH mediated cleavage of the linker by 10-fold (5). Since the rate of cleavage was unaffected by linker length, no further studies were performed on these derivatives.

(c) Conjugation of thioether linked DOTA derivatives to Fab and recombinant antibody fragments. Preliminary studies with radiolabeled Fab or Fab' fragments demonstrated high kidney uptake (up to 200%ID/g) that was not reduced with the chemical labile linker (MC-C6-DOTA; data not shown). Therefore, another linker approach was explored. Since Arano and coworkers (7) had shown that conjugation of mildly reduced Fab fragments to the ε-amino group of a peptide linker with a C-terminal lysine reduced kidney uptake by 2-fold in a radioiodine model, we adapted their approach to radiometals. In order to test this approach, we synthesized a tetrapeptide (GLGK) with DOTA at the N-terminus and lysine at the C-terminus linked to an Fab fragment of cT84.66. Hexane vinyl sulfone was utilized as the linker between the ε-amino group of lysine and the sulfhydryl groups of mildly reduced Fab fragments. The results shown in **Figure 7** demonstrate a four-fold reduction in kidney uptake compared to the active ester DOTA conjugated Fab fragment. Surprisingly, the liver uptake (30 %ID/g) was higher than usual prompting us to explore other antibody fragments. Late in 2001, a recombinant diabody of cT84.66 became available to us (8). The diabody was more attractive that Fab fragments because the diabody has the same molecular weight as a Fab, but is divalent rather than monovalent. Thus, the tumor uptake and retention of diabodies are usually superior to Fab fragments. As a baseline, we determined the kidney uptake of ^{111}In-DOTA-diabody, and as expected, the kidney uptake was in the range of 150-200 %ID/g (8). Therefore, we tested the ability of DOTA-peptide conjugated to diabody to lower kidney uptake. First, it was necessary to design a version of the diabody with a C-terminal cysteine, so that the diabody could be linked through a thioether linkage via the C-terminal lysine of the peptide linker. When this conjugate was labeled with ^{111}In and an animal biodistribution performed (**Figure 8**), the kidney uptake was reduced to 35% ID/g (over 5-fold compared to DOTA-diabody) and the liver uptake was significantly reduced (20 %ID/g, **Figure 8**, vs 30% ID/g, **Figure 7**). Therefore, we conclude that the Arano peptide approach adapted to radiometals is feasible. Thus, a major goal of the grant application has been achieved. A manuscript describing this work is in preparation.

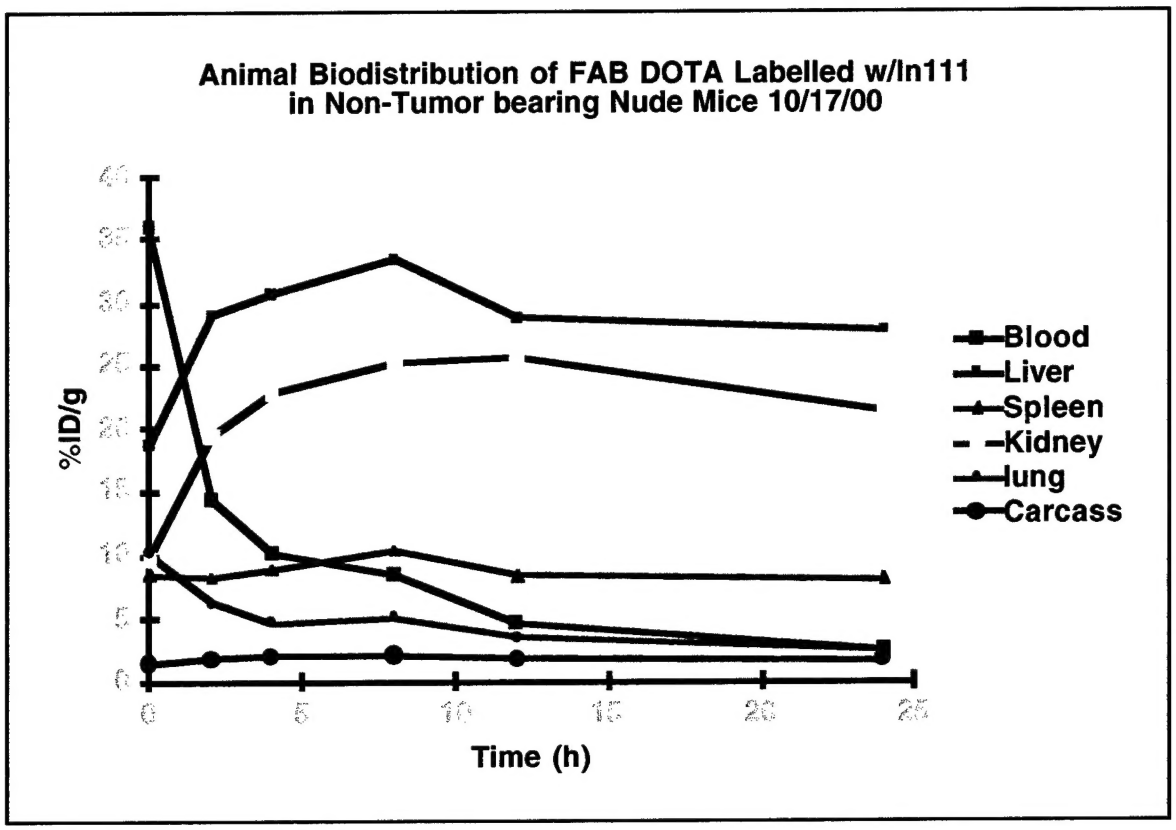


Figure 7. Biodistribution of ^{111}In-DOTA-peptide-Fab in nude mice. Groups of 5 nude mice were injected with 2 uCi (2 ug) of ^{111}In-DOTA-peptide-Fab and tissues analyzed at the indicated time points.

(d) Synthesis and conjugation of novel chelates to antibodies. In the SOW, we proposed the synthesis of an allyl derivative of cys-DOTA with the idea that the different length of the linker would lower normal tissue uptake. Although the allyl derivative was synthesized, it reacted poorly with mildly reduced antibody. We then synthesized several bis-acrylamide derivatives of cys-DOTA and showed that they too conjugated slowly to mildly reduced antibody and gave low yields (<5%) of ^{111}In-labeled DOTA-antibody conjugates. Next, we investigated the ability of vinyl sulfone to form bifunctional derivatives with cys-DOTA. Vinyl sulfone is known to react rapidly with thiols, and as expected, it gave stable derivatives with cys-diabody that conjugated rapidly with mildly reduced antibody. The resulting derivatives gave quantitative labeling with ^{111}In and performed well in animal biodistribution studies (**Figure 9**). We also demonstrated that the vinyl sulfone grup could be directed to either amino or thiol groups by controlling the pH of the reaction conditions (9). Once this work was accomplished, we began to extend the studies to antibody fragments according to the SOW. Since it was already shown that direct conjugation of bifunctional DOTA derivatives to antibody fragments led to high kidney uptake, we inserted the peptide linker between DOTA and the antibody fragment. In these studies we showed that hexane vinyl sulfone rather than vinyl sulfone had to be used. The reason for this is clear- vinyl sulfone forms an unreactive cyclic derivative with the ε–amino group of lysine, while hexane vinyl sulfone does not. Thus, the the overall goals of the project were achieve when we combined the hexane vinyl sulfone chemistry with the Arano linker peptide approach on recombinat cys-diabody (**Figure 8**).

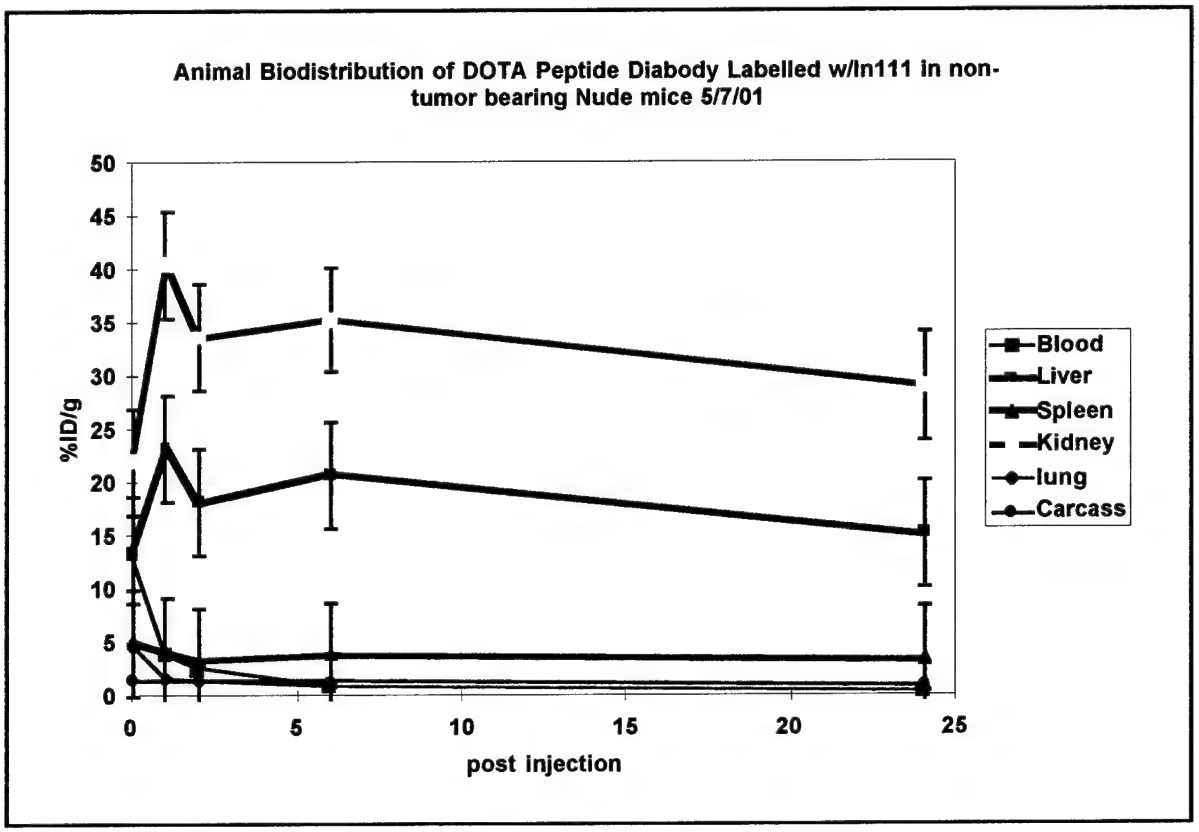


Figure 8. Biodistribution of ^{111}In-DOTA-peptide-cys-diabody in nude mice. Groups of 5 nude mice were injected with 2 uCi (2 ug) of ^{111}In-DOTA-peptide-cys-diabody and tissues analyzed at the indicated time points.

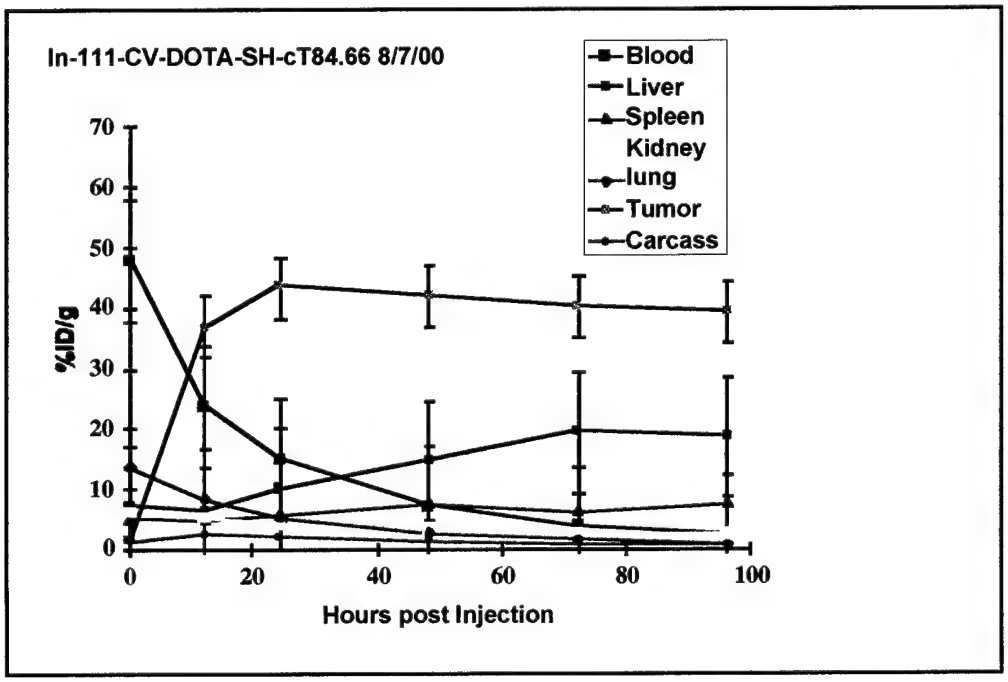


Figure 9. Biodistribution of ^{111}In-vinyl sulfone-cys-DOTA-cT84.66 in nude mice bearing LS174T xenografts. Groups of 5 nude mice bearing LS174T xenografts were injected with 2 uCi (2 ug) of ^{111}In-vinyl sulfone cys-DOTA-cT84.66 and tissues analyzed at the indicated time points.

3.0 Key Research accomplishments

a) We have shown that radiolabeled anti-her2/neu antibodies, either 4D5 or Herceptin, can be used to target and treat tumors that either over-express or express normal amounts of her2/neu in an animal model.

b) We have shown that RIT with either ^{90}Y-labeled anti-CEA or anti-her2/neu antibodies effectively reduce tumor growth in the same animal model. Furthermore, the addition of taxol is probably additive in both systems and improves therapy.

c) We have conjugated peptide derivatives of DOTA to recombinant antibody fragments (diabody or cys-diabody) and shown that they reduce normal kidey uptake up to 5-fold, thus making RIT with recombinant antibodies feasible.

4.0 Reportable outcomes

a) manuscripts:

Tsai, S.W., Sun, YY., Wlliams, L.E., Raubitschek, A.A., Wu, A.M., and Shively, J.E. Biodistribution and radioimmunotherapy of human breast cancer xenografts with radiometal-labeled DOTA conjugated anti-her2/neu antibody 4D5. **Bioconj. Chem.** 11: 327-334, 2000.

Li, L., Tsai, S.-W., Anderson, A.-L., Keire, D.A., Raubitschek., A.A., and Shively, J.E. Viny sulfone bifunctional derivatives of DOTA allow sulfhydryl or amino directed coupling to antibodies. Conjugates retain immunoreactivity and have similar biodistributions. **Bionconj. Chem.** 13: 110-115, 2002.

b) patents: none.

5.0 Conclusions.

The results confirm that the MCF7 tumor xenograft in the nude mouse animal model is suitable for evaluating radioimmunotherapy of ^{90}Y labeled DOTA-derivatives for either anti-her2/neu or anti-CEA antibodies and their recombinant fragments. Novel vinyl sulfone based peptide derivatives of DOTA have been synthesized, conjugated to whole antibodies or recominant fragments and shown to lower normal kidney uptake, thus solving one of the thornier problems preventing the use of radiometal labeled antibody fragments for therapy.

6.0 References.

1. Pietras, R. J., Poen, J. C., Gallardo, D., Wongvipat, P. N., Lee, J., and Slamon, D. J. Monoclonal Antibody to Her-2/*neu* Receptor Modulates Repair of Radiation-induced DNA Damage and Enhances Radiosensitivity of human Breast Cancer Cells Overexpressing This Oncogene, Can. Res. *59:* 1347-1355, 1999.
2. Lewis, M. R., Raubitschek, A., and Shively, J. E. A facile, water-soluble method for modification of proteins with DOTA. Use of elevated temperature and optimized pH to achieve high specific activity and high chelate stability in radiolabeled immunoconjugates., Bioconjugate Chem. *5:* 565-576, 1994.
3. Williams, L. E., Primus, F. J., Wong, J. Y. C., Wu, A. M., Odon-Maryon, T. L., Johnson, D. K., Hefta, L. J. F., Shively, J. E., and Raubitschek, A. A. Biodistribution of an indium-111 or yttrium-90-labelled chimeric anti-carcinoembryonic antigen monoclonal antibody (cT84.66) following its large scale production in a bioreactor, Tumor Targeting. *2:* 116-124, 1996.
4. Tsai, S.W., Sun, YY., Wlliams, L.E., Raubitschek, A.A., Wu, A.M., and Shively, J.E. (2000). Biodistribution and radioimmunotherapy of human breast cancer xenografts with radiometal-labeled DOTA conjugated anti-her2/neu antibody 4D5. *Bioconj. Chem.*, *11* 327-334.

5. Lewis, M. R. and Shively, J. E. Maleimidocysteinyl-DOTA derivatives: new reagents for radiometal chelate conjugation to antibody hinge sulfhydryl groups undergo pH-dependent cleavage reactions. Bioconjugate Chemistry,9:72-86, (1998).
6. Williams, L. E., Lewis, M. R., Bebb, G., Clarke, K. G., Odom-Maryon, T. L., Shively, J. E., and Raubitscheck, A. A. Biodistribution of ^{111}In-labeled and 90Y DOTA and maleimidocysteineamido-DOTA conjugated to chimeric anti-CEA monoclonal antibody in xenograft-bearing nude mice: comparison of stable and chemical labile linker systems. Bioconj. Chem., 9:87-93, (1998).
7. Arano, Y., *et al.*, Chemical design of radiolabeled antibody fragments for low renal radioactivity levels. Cancer Res., 59: 128-134, 1999.
8. Yazaki, P.J., Wu, A.M., Tsai, S.-W., Williams, L.E., Ilke, D.N., Womg, J.Y.C., Shively, J.E., and raubitschek, A.A. Tumor targeting of radiometal labeled anti-CEA recombinant antibody T84.66 diabody and T84.66 minibody: comparison to radiolabeled fragments. Bionconj. Chem. 12, 220-228 (2001).
9. Li, L., Tsai, S.-W., Anderson, A.-L., Keire, D.A., Raubitschek., A.A., and Shively, J.E. Viny sulfone bifunctional derivatives of DOTA allow sulfhydryl or amino directed coupling to antibodies. Conjugates retain immunoreactivity and have similar biodistributions. . Bionconj. Chem. 13, 110-115 (2002).

7.0 Appendices:

See attached manuscripts.

Personnel Receiving Pay on this Grant:

John E. Shively
Lin Li

Biodistribution and Radioimmunotherapy of Human Breast Cancer Xenografts with Radiometal-Labeled DOTA Conjugated Anti-HER2/neu Antibody 4D5

S. W. Tsai, YY. Sun, L. E. Williams, A. A. Raubitschek, A. M. Wu, and J. E. Shively

Division of Immunology, Beckman Research Institute of the City of Hope, Duarte, California 91010, Department of Anatomic Pathology, City of Hope National Medical Center, Duarte, California 91010, Division of Diagnostic Radiology, City of Hope National Medical Center, Duarte, California 91010, Department of Radioimmunotherapy, City of Hope National Medical Center, Duarte, California 91010, and Department of Molecular Biology, Beckman Research Institute of the City of Hope, 1450 East Duarte Road, Duarte, California 91010

Bioconjugate Chemistry®

Reprinted from
Volume 11, Number 3, Pages 327–334

Biodistribution and Radioimmunotherapy of Human Breast Cancer Xenografts with Radiometal-Labeled DOTA Conjugated Anti-HER2/neu Antibody 4D5

S. W. Tsai,[†] YY. Sun,[‡] L. E. Williams,[§] A. A. Raubitschek,[∥] A. M. Wu,[⊥] and J. E. Shively*,[†]

Division of Immunology, Beckman Research Institute of the City of Hope, Duarte, California 91010, Department of Anatomic Pathology, City of Hope National Medical Center, Duarte, California 91010, Division of Diagnostic Radiology, City of Hope National Medical Center, Duarte, California 91010, Department of Radioimmunotherapy, City of Hope National Medical Center, Duarte, California 91010, and Department of Molecular Biology, Beckman Research Institute of the City of Hope, 1450 East Duarte Road, Duarte, California 91010. Received October 1, 1999; Revised Manuscript Received January 19, 2000

HER2/neu oncogene encodes a 185 kDa trans-membrane protein which is overexpressed in 20−30% of breast and ovarian cancers and portends a poor prognosis. We have studied the targeting and therapy of this oncoprotein with 4D5, a murine monoclonal antibody which recognizes a distinct epitope on the extracelluar domain of HER2/neu. We conjugated the antibody with an active ester of the macrocyclic chelating agent DOTA, radiolabeled the conjugate with either ^{111}In or ^{90}Y, and studied the antibody distribution and therapy, respectively, in athymic mice bearing xenografts of MCF7/HER2/neu, a human breast cancer cell line transfected with the HER2/neu oncogene. For the biodistribution of ^{111}In-labeled DOTA-4D5, a high specificity of tumor localization (30% ID/g) was seen with a tumor-to-blood ratio of greater than 2 at 48 h postinjection. Compared to a previously published study with ^{125}I-labeled 4D5 in beige nude mice bearing NIH3T3/HER2/neu xenografts [De Santes et al. (1992) *Cancer Res. 52*, 1916−1923], ^{111}In-labeled 4D5 antibody gave superior antibody uptake in tumor (30% ID/g vs 17% ID/g at 48h). In the therapy study, treatment of the nude mice bearing MCF7/HER2/neu xenografts with 100 μCi (3 μg) of ^{90}Y-labeled DOTA-4D5 caused a 3-fold reduction of tumor growth compared to untreated controls (injected with human serum albumin) in 40 days. Treatment of animals with 100 μCi of nonspecific antibody ^{90}Y-labeled DOTA-Leu16 (3 μg) had no tumor growth inhibition. Treatment with unlabeled DOTA-4D5 (3 μg) had a slight effect on tumor growth compared to untreated controls. When analyzed at the level of single animals, no effect was seen in seven of nine animals; however, in two of the animals, tumor growth inhibition was observed. Although a cold antibody therapeutic effect was unexpected at this dose level (3 μg), it may be possible that in some animals that 3 μg of antibody of ^{90}Y-labeled DOTA-4D5 augmented tumor growth reduction. To further explore the effects of cold antibody treatment alone, animals were treated with 100 or 400 μg of unlabeled 4D5 administered in two doses. These animals showed a 1.7−1.8-fold reduction in tumor growth over 28 days, a result less than that obtained with RIT only.

INTRODUCTION

The HER2/neu receptor, the product of the c-erbB2 oncogene, has been the target of monoclonal antibody clinical therapeutic trials (*1*−*3*). The oncogene encodes a 185 000 transmembrane phosphoglycoprotein (*4*−*6*), is amplified in 20−30% of breast carcinoma (*7*−*9*), and is linked to poor prognosis (*10*). Several studies have suggested that reducing expression of HER2/neu gene product may convey a growth disadvantage on the tumor (*11*, *12*). Many preclinical trials have shown that certain anti-HER2/neu antibodies can inhibit the growth of HER2/neu-overexpressing tumors (*12*−*16*). In particular, the anti-HER2/neu monoclonal antibody 4D5, when used with cisplatin, has been shown to promote drug-induced killing in target cells (*17*, *18*). Recently, Herceptin, the humanized version of the murine 4D5 monoclonal antibody (*19*), was approved by the FDA for the treatment of HER2/neu positive tumors. The approach utilizes a combination of Herceptin and chemotherapeutic agents. In a phase II study in which 37 patients received Herceptin and cisplatin, a 24.3% partial response rate was observed (*2*).

In addition to any biological effects resulting from the direct interaction of anti-HER2/neu antibody with the HER2/neu receptor, anti-HER2/neu antibody can also be used to deliver drugs, toxins, or radioisotopes directly to HER2/neu positive malignant cells. In the case of radioimmunotherapy (RIT),[1] the use of β-emitter radiolabeled antibody is appealing because tumor cells inaccessible to antibody may still be killed by radioimmunoconjugates bound to neighboring cells. DeSantes et al. (*20*) demon-

* To whom correspondence should be addressed. Phone: (626) 301-8301. FAX: (626) 301-8186. E-mail: jshively@coh.org.
[†] Division of Immunology.
[‡] Department of Anatomic Pathology.
[§] Division of Diagnostic Radiology.
[∥] Department of Radioimmunotherapy.
[⊥] Department of Molecular Biology.

[1] Abbreviations: DOTA, 1,4,7, 10-tetraazacyclododecane-*N*,*N*′,*N*″,*N*‴-tetraacetic acid; DTPA, diethylenetriaminepentaacetic acid; FITC, fluorescein isothiocyanate; HSA, human serum albumin; BSA, bovine serum albumin; RIT, radioimmunotherapy; RTV, relative tumor volume; MTD, maximum tolerated dose; ABC, avidin−biotin complex.

10.1021/bc9901292 CCC: $19.00 © 2000 American Chemical Society
Published on Web 03/24/2000

strated that animals treated with 400−700 μCi of ^{131}I-labeled anti-HER2/neu 4D5 monoclonal antibody showed marked inhibition of tumor growth; however, the choice of ^{131}I-labeled antibody requires a higher activity level due to dehalogenation in normal and malignant tissues.

Radiometals that are conjugated to antibody via chelates may be a better choice as exemplified by RIT studies with radiometal labeled anti-lymphoma (*21*), anti-colon cancer (*22*), and anti-breast cancer (*23*) antibodies. Bifunctional chelates such as DTPA (*24*) or DOTA (*25*, *26*) derivatives have been successfully conjugated to antibodies giving products with high retention of immunoreactivities and radiolabeled to high specific activities with either ^{111}In, a γ-emitter with a half-life of 67.9 h, or ^{90}Y, a pure β-emitter with a 64.0 h half-life. In the case of HER2/neu positive tumors, Horak et al. (*27*) have demonstrated good tumor specificity, pharmacokinetics, and radioimmunotherapy with ^{212}Pb-labeled, DOTA-conjugated AE1 anti-HER2/neu antibody in an ovarian tumor model; however, their reagent did not provide effective therapy for large established tumors. In addition, ^{212}Pb may not be suitable for treatment of bulky solid tumors due to its short half-life of 10.6 h and the low tissue penetration of α-rays. Our recent studies have focused on the therapeutic effect of ^{90}Y-labeled, DOTA-conjugated anti-CEA antibodies (*22*). In this report, we have studied the feasibility of targeting the HER2/neu protein with DOTA-conjugated anti-HER2/neu antibody 4D5. Biodistributions of the ^{111}In-labeled and therapy efficacy with the ^{90}Y-labeled antibodies were determined in a nude mouse model bearing the MCF7/HER2/neu xenograft.

EXPERIMENTAL PROCEDURES

Cell Line. MCF7/HER2/neu is a human breast tumor cell line expressing HER2/neu and was obtained from Dr. Dennis Slamon (UCLA). Cells were maintained in RPMI 1640, supplemented with 10% FBS, 100 units/mL penicillin, 100 units/mL streptomycin, and 2 mM L-glutamine. To establish tumor growth in nude mice, subconfluent monolayer cells were trypsinized in EDTA, washed in PBS, and resuspended in RPMI1640.

Antibody. 4D5 is a murine IgG_1 monoclonal directed against the extracellular domains of p185 (HER2/neu) and was a gift from Dr. Paul Carter, Genentech, Inc. The antibody was conjugated with DOTA, using active ester carbodiimide chemistry, with a DOTA-ester-to-antibody ratio of 100:1 (*25*). The antibody was then radiolabeled at 43° C for 1 h with ^{111}In or ^{90}Y, at pH 5. The labeling efficiency was greater than 80% for both radionuclides. The number of chelates per antibody was 5.0 as determined by a modification of a ^{57}Co(II) binding assay (*28*), except $^{111}InCl_3$ was used. The binding affinity of [^{111}In]-DOTA 4D5 to MCF7/HER2/neu cells was 1.25×10^8 M^{-1}, as determined by Scatchard analysis (data not shown). The immunoreactivity of [^{111}In]DOTA 4D5 as determined by binding to MCF7/HER2/neu cells was 96% by linear extrapolation to binding at infinite antigen excess (*29*). The anti-CD20 murine antibody Leu16 (Becton Dickinson) was conjugated with DOTA active ester as described above.

Flow Cytometry Analysis. Specific binding of 4D5 to HER2/neu gene product on MCF7/HER2/neu cells was assessed by indirect immunofluorescence. Untransfected MCF7 cells which do not express HER2/neu were used as a negative control. MCF7/HER2/neu was used as a HER2/neu positive cell line, and Leu16 a nonspecific antibody control. One million cells in 0.5 mL of PBS/1%BSA were incubated with 0.1 μg of antibody for 1 h on ice. The mixtures were then washed two times, incubated with 1 μg of FITC-conjugated goat anti mouse IgG Fab'$_2$ fragment (Jackson Immuno Research Labs) for 40 min, washed once with PBS/1% BSA, and then analyzed on a MoFlo flow cytometer (Cytomation, Fort Collins, CO).

Immunohistochemistry. Fresh tumor tissues were excised from the untreated and from the 100 μCi-4D5 treated group 40 days after therapy. Tissues were embedded in O. C. T. (Miles Inc.) and were quick frozen in isopentane. Tissue sections were cut in 4 um thickness and were mounted on Probe-on slides (Vantana Medical System Inc.). Frozen section slides were fixed in cold acetone for 10 min, then washed with PBS three times, 5 min each. All slides were loaded into a Techmate slide holder and stained using the Biotech Techmate 1000 Immunostainer. Sections were incubated 25 min with 5 μg/mL of 4D5, or with 5 μg/mL of Leu16 as a negative control. The antibody−antigen complex was detected by a modified ABC method (30) (Vantana Medical System Inc.) per manufacturer's direction with the Chromagen 3'3-diaminobenzidine.

Animal Model. Three days prior to injection of tumor cells, 2 month old female mice (NCI) weighing 13-21 g were implanted s.c. with 1.7 mg, 60 day release 17 β-estradiol pellets (Innovative Research of America) on the shoulder pads to promote tumor growth. The mice were inoculated s.c. in the flanks with 10 million MCF7/HER2/neu cells resuspended in 0.15 mL of RPMI1640. Solid tumors measuring approximately 100 mm^3 were visible at 12 days postinoculation. The mice were then randomized according to tumor size to prevent any bias in the biodistribution and radioimmunotherapy studies.

Antibody Biodistribution. Groups of five mice per time point bearing MCF7/HER2/neu xenografts were injected with 3.6 μCi of ^{111}In-labeled DOTA-4D5 via tail vein. Animals were sacrificed at 0, 5, 24, 48, 72, and 96 h postinjection. The average tumor weights were 0.13, 0.25, 0.22, 0.29, 0.35, and 0.41 g, respectively. Tumors were dissected and the major organs and blood weighed and the activity measured. The activity expressed in percentage injected dose per gram of tissue was then calculated. The mean values were used to construct biodistribution curves for tumors and normal organs.

Radioimmunotherapy. DOTA-4D5 and DOTA-Leu 16 were labeled with ^{90}Y to specific activities of 33.3 μCi/μg. Groups of nine tumor-bearing mice (average volume 100 mm^3) were injected via tail vein with 50 μCi or 100 μCi DOTA-4D5, 100 μCi DOTA-Leu16, DOTA-4D5, or 1% HSA/saline. Each mouse received equal amounts of protein (3 μg). Tumors were measured twice a week (after antibody injection) for 37 days. Tumor volume was calculated as Length × Width^2/2, where length was the longer of the two measurements. The relative tumor volume was calculated as the ratio of tumor volume on that day to its value at the start of therapy. The growth curve was plotted as the average of the relative tumor volume within a group vs time.

To assess the effect of unlabeled 4D5 on tumor growth, groups of eight tumor-bearing mice (average tumor volume 200 mm^3) were also given 100 μg or 400 μg of 4D5 in two doses by i.p. injection on day zero and on day eight. Leu16 (400 μg) in two doses was used as a negative control. Tumor volumes were measured for 28 days, and the growth curves were constructed as above.

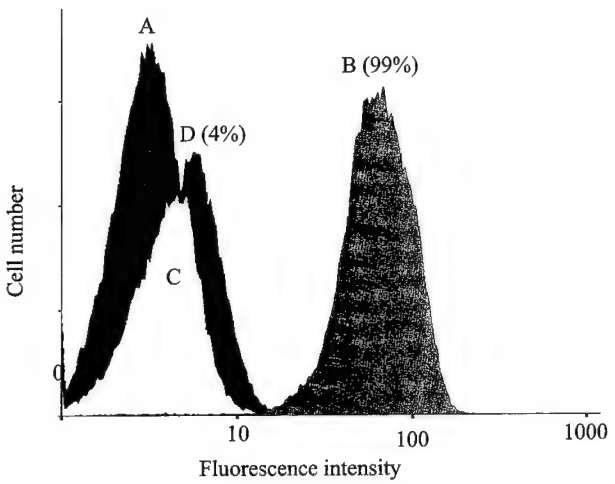


Figure 1. Fluorescent-activated cell sorter analysis of binding of 4D5 to MCF7/HER2/neu and to MCF7 cells. (A) MCF7/HER2/neu control, stained with Leu16, (B) MCF7/HER2/neu stained with 4D5, (C) MCF7 control stained with no primary antibody, (D) MCF7 stained with 4D5. The percent positive cells (vs control) are shown for B and D.

RESULTS

Antibody and Cell Analysis. Indirect immunofluorescence with flow cytometry demonstrated intense, specific binding of the DOTA-4D5 antibody to MCF7/HER2/neu cells (99% positive vs control), but low binding to the parent MCF7cell line (4% vs control), a cell line with limited expression of the HER-2/neu gene product (Figure 1). The 4D5 antibody was conjugated to DOTA, a macrocyclic chelator, using an active ester chemical method (*25*). The DOTA-4D5 conjugate was shown to have a DOTA/antibody ratio of 5/1, radiolabeled with either ^{111}In or ^{90}Y to high efficiency (>90%), and retained high immunoreactivity (>90%) when tested in a cell-binding assay. Nude mice bearing MCF7/HER2/neu xenografts were used as a model for tumor targeting with radiolabeled DOTA-4D5. Immunohistochemical analysis of HER2/neu expression in both treated and nontreated tumors showed membrane staining in approximately 99% of the tumor cells, while staining with the control Leu16 antibody showed only background staining (Figure 2). These results demonstrate that the HER2/neu xenografts continue to produce HER2/neu on the cell surface while growing in nude mice, including after treatment with [^{90}Y]DOTA-4D5.

Antibody Biodistribution. The biodistribution of [^{111}In]DOTA-4D5 was evaluated in nude mice bearing MCF7/HER2/neu xenografts. Groups of five mice per time point were injected with 3.6 μCi of [^{111}In]DOTA-4D5 via the tail vein. Biodistribution studies performed with [^{111}In]DOTA-4D5 showed progressive accumulation of the antibody in MCF7/HER2/neu tumors ranging from 8% ID/g at 5 h to 24% ID/g at 96 h postinjection, as shown in Figure 3 and Table 1 of the Supporting Information. At the same time points, the activity present in blood decreased from 23% to 10% ID/g. The tumor-to-blood ratio increased from 0.36 at 5 h to 2.4 at 96 h postinjection. Organs with significant nonspecific accumulations of antibody included the liver, kidneys, and lung, which ranged from 6 to 11% ID/g at 5 h; however, the accumulations decreased to less than 5% ID/g at 96 h. The results demonstrated specific accumulation of antibody in tumors compared to other major organs.

Radioimmunotherapy. To assess the therapeutic efficacy of DOTA-4D5 in athymic mice bearing MCF7/HER2/neu xenografts, we injected 50 or 100 μCi of ^{90}Y-labeled DOTA-4D5 (3 μg) into groups of nine mice. Control groups were injected with 100 μCi of ^{90}Y-labeled DOTA-Leu16 (3 μg), unlabeled DOTA-4D5 (3 μg), or 1% human serum albumin in saline. With the exception of the HSA control group, all groups received a single injection of 3 μg of antibody. Tumor volumes were measured twice weekly. The average relative tumor volume (RTV) was calculated as the ratio of tumor volume on that day to the volume at the start of therapy. The average relative tumor volume within each group was compared over 37 days (Figure 4). Mice injected with 50 μCi ^{90}Y-labeled DOTA-4D5 showed a 2-fold reduction in tumor growth at day 37, as compared to mice injected with serum albumin/saline [RTV 4.69 vs 9.30, $p < 0.04$, t-test; Table 2 (Supporting Information)]. When the

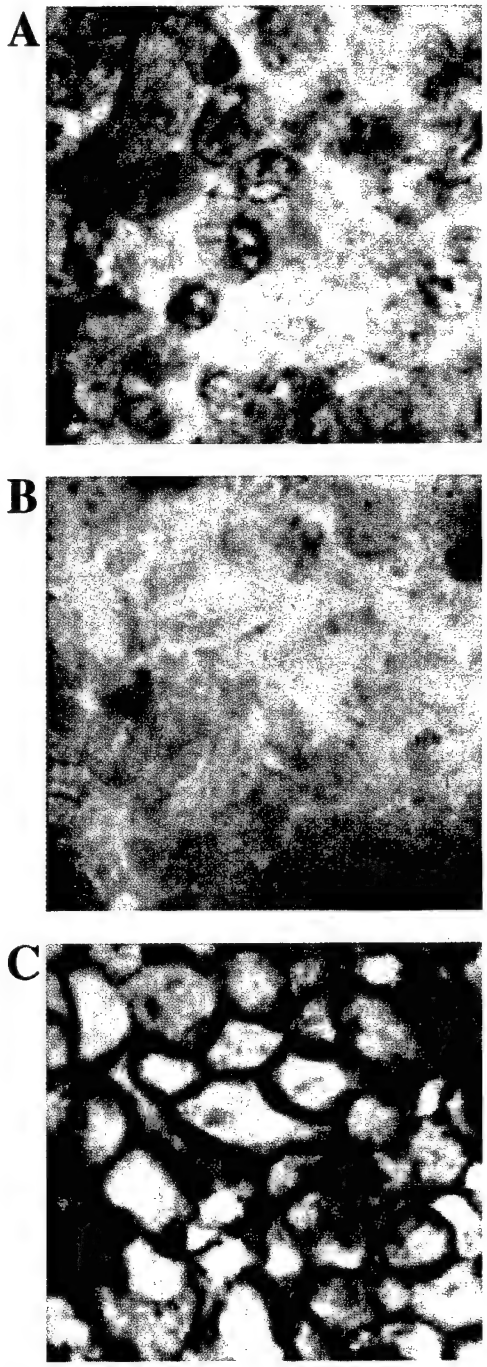


Figure 2. Immunohistochemical frozen sections of MCF7/HER2/neu tumor excised from untreated mice 40 days after therapy. Tumor frozen sections probed with (A) no primary antibody, (B) Leu16 (control), and (C) 4D5.

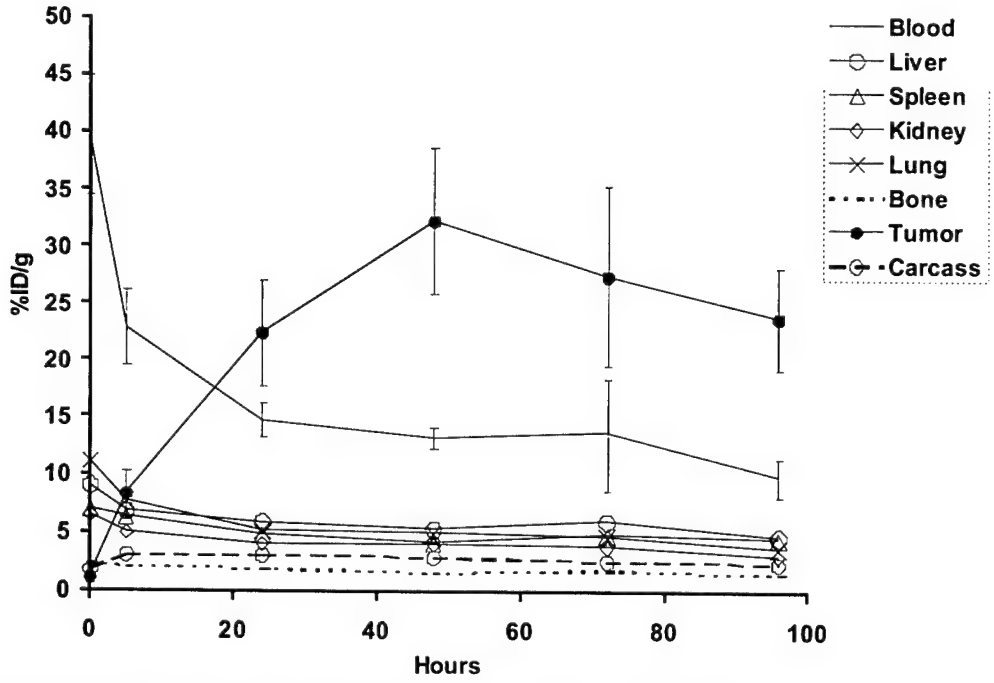


Figure 3. Biodistribution of ^{111}In-labeled DOTA-4D5 in nude mice bearing MCF7/HER2/neu xenografts. Groups of five mice were injected with 3.6 μCi (2.4 μg) of ^{111}In-labeled DOTA-4D5. At 0, 5, 24, 48, 72, and 96 h postinjection the tumors and normal organs were excised, weighed and the ^{111}In activity measured. The mean %ID/g (±std dev) at each time point are shown for blood, liver, spleen, kidney, lung, bone, tumor, and carcass.

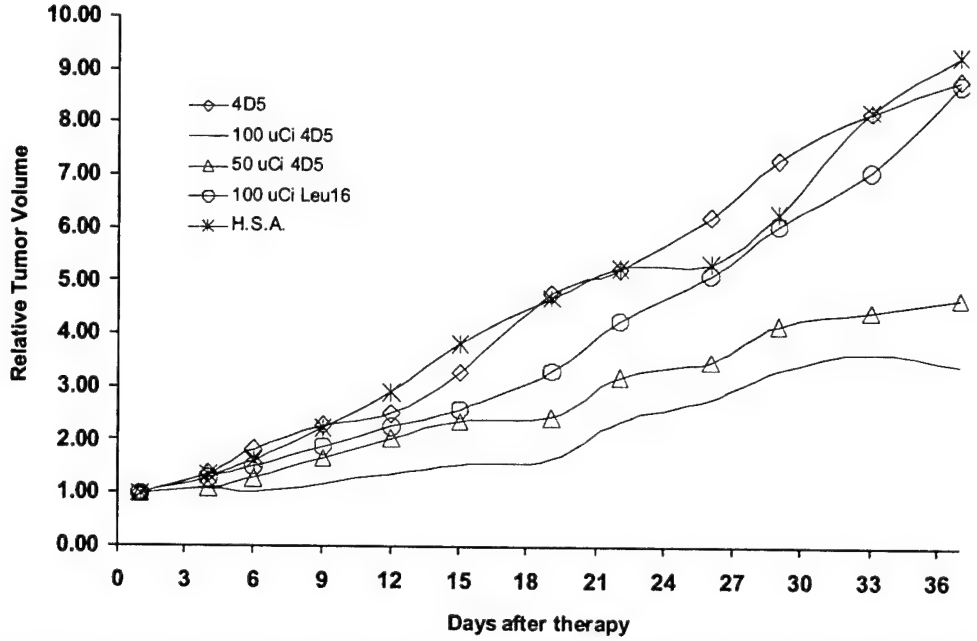


Figure 4. Radioimmunotherapy of established MCF7/HER2/neu tumors in nude mice with ^{90}Y-labeled DOTA-4D5. Groups of nine mice were injected i.v. with (◇) 3 μg of unlabeled DOTA-4D5, (—) 100 μCi (3 μg) of ^{90}Y-labeled DOTA-4D5, (△) 50 μCi (3 μg) of ^{90}Y-labeled DOTA-4D5, (○) 100 μCi (3 μg) of ^{90}Y-labeled DOTA-Leu16, or (*) 1% HSA/saline. The tumor volume for each mouse at the time indicated was normalized to the tumor volume at the start of treatment. Mean values (±std dev) for all animals in each group are shown in Table 2 (Supporting Information).

amount of activity was increased to 100 μCi, the retardation of tumor growth reached 2.7-fold (RTV 3.42 vs 9.30, $p < 0.02$, t-test). Treatment with ^{90}Y-labeled control antibody, DOTA-Leu16, however, did not slow tumor growth over the course of study (RTV 8.77 vs 9.30). Similarly, treatment with unlabeled DOTA-4D5 did not result in significant tumor growth retardation compared to the RIT-treated animals (RTV 8.83 vs 9.30). However, if the data is analyzed at the level of single animals, we noted that two of nine animals treated with unlabeled DOTA-4D5 showed tumor growth inhibition (Table 2, Supporting Information). If a single animal had shown this result, we might have dismissed it; however, the finding occurred in two animals and the effect was not seen in the serum/albumin/saline-treated controls.

To assess the effect of cold antibody alone on tumor growth, we treated mice with higher levels of unlabeled 4D5 (Figure 5). Mice injected with 100 μg of 4D5 showed a 1.65-fold reduction in tumor growth at day 28, as compared to mice injected with 400 μg of Leu16 (RTV 4.09 vs 6.75, $p = 0.15$, t-test; Table 1). Mice injected with 400 μg of 4D5 showed a 1.80-fold reduction (RTV 3.74 vs

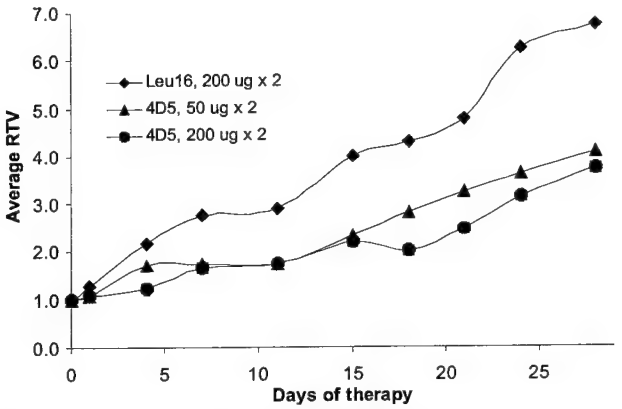


Figure 5. Immunotherapy of established MCF7/HER2/neu tumors in nude mice with 4D5. Groups of eight mice were injected i.p. on day zero and on day eight with (▲) 50 μg of 4D5, (●) 200 μg of 4D5, or (◆) 200 μg of Leu16. The tumor volume for each mouse at the time indicated was normalized to the tumor volume at the start of treatment. Mean values (±std dev) for all animals in each group are shown in Table 1.

6.75, $p = 0.11$, t-test). On the basis of these observations, we concluded that even at the level of 3 μg of antibody treatment, the majority of the RIT effect is due to radiation but not to the cytotoxic effects of antibody alone. Thus, despite the unexpected effect of cold antibody, our conclusions remain the same, i.e., that RIT treatment with the HER2/neu specific antibody has a statistically significant effect on tumor growth compared to the unlabeled antibody-treated group. Finally, it should be noted that while [^{90}Y]DOTA-4D5 slowed tumor growth, tumor regrowth occurred after about 19 days after therapy, as shown in Figure 4. Statistical analysis (ANOVA single factor) showed that there was no statistically significant difference among the five groups after 19 days.

DISCUSSION

To use HER2/neu as a target for radioimmunotherapy, it is necessary to show that an anti-HER2/neu antibody can be successfully radiolabeled without loss of immunoreactivity, that the radiolabeled antibody can target to HER2/neu positive tumors with good tumor to normal tissue ratios, and that RIT shows a positive effect compared to appropriate controls in a preclinical model. In this study, we utilized the previously described MCF7/HER2/neu cell line (*31*) as a model system. This cell line is derived from MCF7, a commonly used estrogen-sensitive cell line in tumor models of breast cancer. The cell line grows well in nude mice bearing slow estrogen release implants and, as shown in Figure 1, reacts strongly with the anti-HER2/neu antibody 4D5. In addition, HER2/neu expression was demonstrated in both treated and untreated tumors in the nude mouse xenograft model (Figure 2). We chose the monoclonal antibody 4D5 because its reactivity with HER2/neu is well established (*18*, *20*, *31*–*33*) and its humanized counterpart, Herceptin, is approved for clinical use. Furthermore, we were able to conjugate 4D5 with the macrocylic chelator DOTA and radiolabel it with ^{111}In to high specific activity (>30 μCi/μg, 90% labeling efficiency) while retaining high immunoreactivity (>90%) and good targeting to the MCF7/HER2/neu xenografts in nude mice (Figure 3, Table 1 of the Supporting Information). In comparing our results to those of De Santes et al. (*20*) who used the HER2/neu-transfected cell line NIH3T3/HER2/neu in beige/nude mice and ^{125}I-labeled 4D5, we obtained a higher maximum tumor uptake (30% ID/g vs 17% ID/g) and similar tumor to normal tissue ratios at 48h (e.g., tumor/liver uptake for ^{125}I-labeled 4D5 was 6.3, and for ^{111}In-labeled 4D5, the ratio was 6.1). The better tumor uptake may have been due to either dehalogenation of the ^{125}I-labeled antibody in tumor or to the better expression of HER2/neu in the MCF7/HER2/neu xenograft model. The former effect is more likely and has been shown to be a major advantage of radiometal labeled antibodies over radioiodine labeled antibodies (*34*–*37*). The favorable biodistibutions prompted us to perform RIT in the same model with ^{90}Y-labeled DOTA-4D5.

RIT with ^{90}Y-labeled DOTA-4D5 showed a significant tumor growth inhibitory effect compared to untreated (serum albumin/saline) controls, antibody only controls, or ^{90}Y-labeled DOTA-Leu16 controls (Figure 4 and Table 2 of the Supporting Information). The growth inhibitory effect was seen over a period of 19 days, after which tumor regrowth occurred and there was no further significant difference in growth rates among the four groups. In this model, where a single dose of RIT was given, positive effects of the treatment lasted over 6 half-lives of the isotope used (^{90}Y, 64 h). It is possible that a stronger effect would have been obtained if the animals were retreated or if a larger dose of radioactivity were administered. In this respect, we found a stronger effect for 100 μCi/animal compared to 50 μCi/animal (RTV 3.42 vs 4.69). Since no animals died with the 100 μCi dose of RIT, it may be possible to administer even higher doses. In previous studies using ^{90}Y-labeled anti-CEA antibodies in a colon cancer model with anti-CEA antibodies, we found an MTD of 120–130 μCi, which could be increased to 200 μCi if the animals were supported with bone marrow stem cells (*38*). Since the major toxicity in studies of this kind are hematologic and due to circulating radiolabeled antibody, the calculation of the appropriate highest dose depends on blood levels of the antibody (which differ depending on the tumor antigen and antibody system). In this study, several of the animals in the control (^{90}Y-labeled Leu16) RIT group died, since the circulating levels of ^{90}Y-labeled DOTA-Leu16 were higher than for ^{90}Y-labeled DOTA-4D5. The difference in circulating levels between the two antibodies is due to tumor uptake and possibly antigen–antibody clearance to the liver in the one case, but not in the other. The latter possibility cannot be verified, since soluble HER2/neu levels weren't measured.

In the RIT studies, we included an unlabeled antibody control group in which antibody was administered at the same protein dose (3 μg, 0.1 mg/kg) as in the RIT-treated animals. Since this dose was well below what is considered to be a therapeutic level in other studies with this cell line (*31*), we expected no antitumor effects. However, two of nine animals showed significant tumor growth inhibition. In one animal, the tumor was very small (34 mm^3), while the tumor was larger (75 mm^3) in the second animal. Even with these animals included, there was a significant difference between this control group and the RIT-treated groups until 19 days ($p < 0.02$, ANOVA single factor). If these two animals are excluded from this control group, the difference among the RIT groups is even greater ($p < 0.01$ at 19 days and $p < 0.04$ at 37 days, ANOVA single factor). Since the effect was seen for two animals, we doubt that the effect represents a random biological variance. In fact, in a separate experiment, we found that antibody doses 30 times higher (100 μg) had definite antitumor effects (Figure 5 and Table 1). Furthermore, in a slightly different model system, Baselga et al. (*39*) observed antitumor effects at multiple

Table 1. Average Relative Tumor Volumes vs Days after Therapy[a]

treatment	days of therapy									
	0	1	4	7	11	15	18	21	24	28
4D5, 50 μg × 2	1.00	1.06 (0.25)	1.71 (0.44)	1.75 (0.47)	1.74 (0.82)	2.32 (1.23)	2.82 (1.68)	3.24 (2.03)	3.62 (2.36)	4.09 (2.86)
4D5, 200 μg × 2	1.00	1.08 (0.36)	1.24 (0.52)	1.65 (0.61)	1.73 (0.63)	2.20 (0.60)	2.02 (0.50)	2.47 (0.86)	3.13 (1.22)	3.74 (1.66)
Leu16, 200 μg ×2	1.00	1.28 (0.25)	2.16 (1.43)	2.76 (1.62)	2.89 (1.80)	4.01 (2.90)	4.29 (2.89)	4.77 (3.36)	6.25 (5.75)	6.75 (6.10)

[a] Groups of eight mice bearing MCF7/HER2/neu xenografts were injected on day zero and on day 8 with 50 μg of 4D5, 200 μg of 4D5, or 200 μg of Leu16. Mean value (±std dev) for all animals in each group are shown.

antibody doses in the 0.1 mg/kg range. However, using the same cell line as in our study (MCF7/HER2/neu) and the same antibody (4D5), Pietras et al. (*31*) found that antibody doses 100 times higher were required to produce some antitumor effects. Overall, we believe that a small single dose of antibody was not responsible for the antitumor effect in our model RIT system.

In the RIT studies reported by De Santes and coworkers (*20*), ^{131}I-labeled 4D5 was used to treat NIH 3T3/HER2/neu xenografts in beige/nude mice. Significant tumor growth inhibition was observed out to 28 days with regrowth beginning after day 20. The activity required for maximum effect was 400 μCi, compared to 100 μCi in our study. Significant growth inhibition was also observed for the ^{131}I-labeled irrelevant antibody control, probably due to the high nonspecific doses characteristic of ^{131}I. It is also known that ^{131}I-labeled 4D5 is rapidly internalized in HER2/neu positive cells, followed by dehalogenation and excretion of the radioiodine (*20*). Metabolism of radiometal-labeled antibodies is much slower, allowing accumulation of radiomental within the cell and thus exposure of the cell to higher doses of radiation (*35*, *40*–*41*). Other differences between the two studies can be ascribed to the differences in growth rates of the two cell lines in the xenograft model. In untreated controls, the NIH3T3/HER2/neu tumor reached 10 cm^3 in 15 days compared to only 1.0 cm^3 in 40 days for the MCF7/HER2/neu model. It is likely that the slower growing tumors represent a more realistic model for human breast cancer and that their response to therapy is accordingly lower.

In the RIT studies reported by Horak et al. (*27*), ^{212}Pb-labeled DOTA-conjugated anti-HER2/neu antibody AE1 was used to treat nude mice bearing HER2/neu positive SK-OV3 xenografts. When small tumors (average mean size 15 mm^3) were treated, significant growth inhibition out to 60 days was observed with 10 or 20 μCi of radiolabeled antibody compared to untreated controls or ^{212}Pb-labeled DOTA-anti-TAC (irrelevant antibody control). In their study, tumor regrowth occurred at 60–70 days. When larger tumors were studied (average mean size 146 mm^3), tumor growth inhibition was not observed, suggesting that α-emitters are more effective against smaller tumors, a result in keeping with the short path length of α-emitters (several cell diameters). On the other hand, α-emitters deposit more energy per μCi (6–9 vs 1–2 MeV for ^{90}Y) thus allowing one to use less activity than ^{90}Y for a given experiment. In our study, the mean tumor sizes were approximately 100 mm^3 at the beginning of RIT and a tumor inhibitory effect was observed for ^{90}Y-labeled mAb.

Our preliminary study showed promising results in favor of using radiometal-labeled-anti HER2/neu antibody for treating breast carcinoma. We have shown that the antibody is readily conjugated to DOTA and radiolabeled without loss of immunoreactivity, and that the radiolabeled antibody targets to HER2/neu positive tumors with good tumor to normal tissue ratios, and that RIT shows tumor growth reduction compared to appropriate controls in a preclinical model. However, several issues need to be addressed in order to increase the therapeutic potential. First, although in our therapy groups the tumor growth is significantly reduced, tumor regrowth occurs after 19 days, and few mice were cured. Increasing radiation dose level or giving multiple doses may increase the therapeutic efficacy of the radiometal-labeled antibody. Second, given that even a small amount of cold antibody treatment alone may slow tumor growth, a combination therapy of radiometal-labeled antibody and unlabeled antibody may have additive or synergistic effects on tumor growth reduction. We are currently investigating these possibilities.

ACKNOWLEDGMENT

We thank Anne-Line Anderson and Randall Woo for their technical assistance, Stephanie Wright and Jianyi Yuan for their assistance in animal work. This work was funded by the Army Medical Research and Materiel Command Grant DAMD17-98-1-8031.

Supporting Information Available: Tables showing biodistribution of ^{111}In-labeled DOTA-4D5 in nude mice bearing MCF7/HER2/neu xenografts and average relative tumor volume vs days after therapy. This material is available free of charge via the Internet at http://pubs.acs.org.

LITERATURE CITED

(1) Baselga, J., Tripathy, D., Mendelsohn, J., Baughman, S., Benz, C. C., Dantis, l., Sklarin, N. T., Seidman, A. D., Hudis, C. A., Moore, J., Rosen, P. P., Twaddell, T., Henderson, I. C., and Norton, L. (1996). Phase II study of weekly intravenous recombinant humanized anti-p185^{HER2} monoclonal antibody in patients with HER2/neu-overexpressing metastatic breast cancer. *J. Clin. Oncol. 14*, 737–744.

(2) Pegram, M. D., Lipton, A., Hayes, D. F., Weber, B. L., Baselga, J. M., Tripathy, D., Baly, D., Baughman, S. A., Twaddell, T., Glaspy, J. A., and Slamon, D. J. (1998) Phase II study of receptor-enhanced chemosensitivity using recombinant humanized anti-p185$^{HER2/neu}$ monoclonal antibody plus cisplatin in patients with HER2/*neu*-overexpressing metastic breast cancer refractory to chemotherapy treatment. *J. Clin. Oncol. 16*, 2659–2671.

(3) Valone, F. H., Kaufman, P. A., Guyre, P. M., Lewis, L. D., Memoli, V., Deo, Y., Graziano, R., Fisher, J. L., Meyer, L., and Mrozek-Orlowski, M. (1995) Phase Ia/Ib trial of bispecific antibody MDX-210 in patients with advanced breast or ovarian cancer that overexpresses the proto-oncogene HER-2/neu. *J. Clin. Oncol. 13*, 2281–2292.

(4) Bargmann, C. I., Hung, M.-C., and Weinberg, R. A. (1986) The neu oncogene encodes an epidermal growth factor receptor-related protein. *Nature 319*, 226–230.

(5) Coussens, L., Yang-Feng, T. L., Liao, Y.-C., Chen, E., Gray, A., McGrath, J., Seeberg, P. H., Libermann, T. A., Schlessing-

er, J., Francke, U., Levison, A., and Ulrich, A. (1985) Tyrosine kinase receptor with extensive homology to EGF receptor shares chromosomal location with neu oncogene. *Science 230*, 1132–1139.

(6) Schechter, A. L., Stern, D. F., Vaidyanathan, L., Decker, S. J., Drebin, J. A., Greene, M. I., and Weinberg, R. A. (1984) The neu oncogene: an erb-B related gene encoding a 185,-000 Mr tumor antigen. *Nature 312*, 513–516.

(7) Iglehart, J. D., Kraus, M. H., Langton, B. C., Huper, G., Kerns, B. J., and Marks, J. R. (1990) Increased erb B-2 gene copies and expression in mulitple stages of breast cancer. *Cancer Res. 50*, 6701–6707.

(8) King, C. R., Kraus, M. H., and Aaronson, S. A. (1985) Amplification of a novel v-erb B-related gene in a human mammary carcinoma. *Science 229*, 974–976.

(9) Slamon, D. J., Godolphin, W., Jones, L. A., Holt, J. A., Wong, S. G., Keith, D. E., Levin, W. J., Stuart, S. G., Udove, J., Ulrich, A., and Press, M. F. (1989) Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. *Science 244*, 707–712.

(10) Slamon, D. J., Clark, G. M., Wong, S. G., Levin, W. J., Ulrich, A., and McGuire, W. L. (1987) Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. *Science 235*, 177–182.

(11) Drebin, J. A., Link, V. C., Stern, D. F., Weinberg, R. A., and Greene, M. I. (1985) Down-modulation of an oncogene protein product and reversion of the transformed phenotype by monoclonal antibodies. *Cell 41*, 695–706.

(12) Drebin, J. A., Link, V. C., Weinberg, R. A., and Greene, M. I. (1986) Inhibition of tumor growth by a monoclonal antibody reactive with an oncogene-encoded tumor antigen. *Proc. Natl. Acad. Sci. U.S.A. 83*, 9129–9133.

(13) Hancock, M. C., Langton, B. C., Chan, T., Toy, P., Monahan, J. J., Mischak, R. P., and Shawver, L. K. (1991) A monoclonal antibody against the c-erb B-2 protein enhances the cytotoxicity of cis-diamminedichloroplatinum against human breast and ovarian cell lines. *Cancer Res. 51*, 4575–4580.

(14) Hudziak, R. M., Lewis, G. D., Winget, M., Fendly, B. M., Shepard, H. M., and Ulrich, A. (1989) p185HER2 monoclonal antibody has antiproliferative effects in vitro, and sensitizes human breast tumor cells to tumor necrosis factor. *Mol. Cell Biol. 9*, 1165–1172.

(15) Lewis, G. D., Figari, I., Fendly, B., Wong, W. L., Carter, P., Gorman, C., and Shepard, H. M. (1993) Differential responses of human tumor cell lines to anti-p185^{HER2} monoclonal antibodies. *Cancer Immunol. Immunother. 37*, 255–263.

(16) Stancovski, I., Hurwitz, E., Leitner, O., Ulrich, A., Yarden, Y., and Sela, M. (1991) Mechanistic aspects of the opposing effects of monoclonal antibodies to the erbB-2 receptor on tumor growth. *Proc. Natl. Acad. Sci. U.S.A. 88*, 8691–8695.

(17) Arteaga, C. L., Winnier, A. R., Poirier, M. C., Lopez-Larraza, D. M., Shawver, L. K., Hurd, S. D., and Stewart, S. J. (1994) p185c-erbB-2 signal enhances cisplatin induced cytoxicity in human breast carcinoma cells: association between an oncogenic tyrosine kinase and drug-induced DNA repair. *Cancer Res. 54*, 3758–3765.

(18) Pietras, R. J., Fendly, B. M., Chazin, V. R., Pegram, M. D., Howell, S. B., and Slamon, D. J. (1994) Antibody to HER-2/neu receptor blocks DNA repair after cisplatin in human breast and ovarian cancer cells. *Oncogene 9*, 1829–1838.

(19) Carter, P., Presta, L., Groman, C. M., Ridgway, J. B. B., Henner, D., Wong, W. L. T., Rowland, A. M., Kotts, C., Carver, M. E., and Shepard, H. M. (1992) Humanization of an anti-p185^{HER2} antibody for human cancer therapy. *Proc. Natl. Acad. Sci. U.S.A. 89*, 4285–4289.

(20) De Santes, K., Slamon, D., Anderson, S. K., Shepard, M., Fendly, B., Maneval, D., and Press, O. (1992) Radiolabeled antibody targeting of the HER-2/*neu* oncoprotein. *Cancer Res. 52*, 1916–1923.

(21) DeNardo, S. J., DeNardo, G. L., Kukis, D. L., Shen, S., Kroger, L. A., DeNardo, D. A., Goldstein, D. S., Mirick, G. R., Salako, Q., Mausner, L. F., Srivastava, S. C., and Meares, C. F. (1999) 67Cu-2IT-BAT-Lym-1 pharmacokinetics, radiation dosimetry, toxicity, and tumor regression in patients with lymphoma. *J. Nucl. Med. 40*, 302–310.

(22) Wong, J. Y., Williams, L. E., Yamauchi, D. M., Odom-Maryon, L., Esteban, J. M., Neumaier, M., Wu, A. M., Johnson, D. K., Primus, F. J., Shively, J. E., and Raubitschek, A. A. (1995) Initial experience evaluating 90-yttrium radiolabeled anti-CEA chimeric T84.66 in a phase I radioimmunotherapy trial. *Cancer Res. 55*, 5929s–5934s.

(23) DeNardo, S. J., Kramer, E. L., O'Donnell, R. T., Richman, C. M., Salako, Q. A., Shen, S., Noz, M., Glenn, S. D., Ceriani, R. L., and DeNardo, G. L. (1997) Radioimmunotherapy for breast cancer using indium-111/yttrium-90 BrE-3: results of a phase I clinical trial. *J. Nucl. Med. 38*, 1180–1185.

(24) Brechbiel, M. W., Gransow, O. A., Atcher, R. W., Schlom, J., Esteban, J., Simpson, D. E., and Colcher, D. (1986) Synthesis of 1-(p-Isothiocyanatobenzyl) derivatives of DTPA and EDTA. antibody labeling and tumor-imaging studies. *Inorg. Chem. 25*, 2772–2781.

(25) Lewis, M. R., Raubitschek, A., and Shively, J. E. (1994) A facile, water-soluble method for modification of proteins with DOTA. Use of elevated temperature and optimized pH to achieve high specific activity and high chelate stability in radiolabeled immunoconjugates. *Bioconjugate Chem. 5*, 565–576.

(26) Li, M., Meares, C. F., Zhong, G.-R., Miers, L., Xiong, C.-Y., and DeNardo, S. J. (1994) Labeling of monoclonal antibodies with ^{90}Yttrium- and ^{111}Indium-DOTA chelates: A simple and efficient method. *Bioconjugate Chem. 5*, 101–104.

(27) Horak, E., Hartmann, F., Garmestani, K., Wu, C., Brechbiel, M., Gansow, O. A., Landolfi, N. F., and Waldmann, T. A. (1997) Radioimmunotherapy targeting of HER2/neu oncoprotein on ovarian tumor using Lead-212-DOTA-AE1. *J. Nuclear Med. 38*, 1944–1950.

(28) Meares, C. F., McCall, M. J., Reardan, D. T., Goodwin, D. A., Diamanti, C. I., and McTigue, M. (1984) Conjugation of antibodies with bifunctional chelating agents: Isothiocyanate and bromoacetamide reagents, Methods of Analysis, and Subsequent Addition of Metal Ions. *Anal. Biochem. 142*, 68–78.

(29) Lindmo, T., Boven, E., Cuttitta, F., Fedorko, J., and Bunn, P. A., Jr. (1984) Determination of the immunoreactive fraction of radiolabeled monoclonal antibodies by linear extrapolation to binding at infinite antigen excess. *J. Immun. Methods 72*, 77–89.

(30) Hsu, S. M., Raine, L., and Fanger, H. (1981) Use of avidin–biotin-peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabeled antibody (PAP) procedures. *J. Histochem. Cytochem. 29*, 577–580.

(31) Pietras, R. J., Poen, J. C., Gallardo, D., Wongvipat, P. N., Lee, H. J., and Slamon, D. J. (1999) Monoclonal antibody to HER-2/neureceptor modulates repair of radiation-induced DNA damage and enhances radiosensitivity of human breast cancer cells overexpressing this oncogene. *Cancer Res. 15*, 1347–1355.

(32) Fendly, B. M., Winget, M., Hudziak, R. M., Lipari, M. T., Napier, M. A., and Ulrich, A. (1990) Characterization of murine monoclonal antibodies reactive to either the human epidermal growth factor receptor or HER2/neu gene product. *Cancer Res. 50*, 1550–1558.

(33) Pietras, R. J., Pegram, M. D., Finn, R. S., Maneval, D. A., and Slamon, D. J. (1998) Remission of human breast cancer xenografts on therapy with humanized monoclonal antibody to HER-2 receptor and DNA-reactive drugs. *Oncogene 17*, 2235–2249.

(34) Brown, B. A., Comeau, R. D., Jones, P. L., Liberatore, F. A., Neacy, W. P., Sands, H., and Gallagher, B. M. (1987) Pharmacokinetics of the monoclonal antibody B72.3 and its fragments labeled with either ^{125}I or ^{111}In. *Cancer Res. 47*, 1149–1154.

(35) Schott, M. E., Milenic, D. E., Yakota, T., Whitlow, M., Wood, J. F., Fordyce, W. A., Cheng, R. C., and Schlom, J. (1992) Differential metabolic patterns of iodinated versus radiometal chelated anticarcinoma single-chain Fv molecules. *Cancer Res. 52*, 6413–6417.

(36) Sharkey, R. M., Blumenthal, R. D., Behr, T. M., Wong, G. Y., Haywood, L., Forman, D., Griffiths, G. L., and Goldenberg,

D. M. (1997) Selection of radioimmunoconjugates for the therapy of well established or micrometastatic colon carcinoma. *Int. J. Cancer 72*, 477–485.

(37) Stein, R., Chen, S., Haim, S., and Goldenberg, D. M. (1997) Advantage of yttrium-90-labeled over iodine-131-labeled monoclonal antibodies in the treatment of a human lung carcinoma xenograft. *Cancer 80*, 2636–2641.

(38) Morton, B. A., Beatty, B. G., Mison, A. P., Wanek, P. M., and Beatty, J. D. (1990) Role of bone marrow transplantation in ^{90}Y antibody therapy of colon cancer xenografts in nude mice. *Cancer Res.* (Suppl.) *50*, 1008s–1010s.

(39) Baselga, J., Norton, L., Albanell, J., Kim, Y., and Mendelsohn, J. (1998) Recombinant humanized anti-HER2 antibody (Herceptin™) enhances the antitumor activity of Paclitaxel and Doxorubicin against HER2/*neu* overexpressing human breast cancer xenografts. *Cancer Res. 58*, 2825–2831.

(40) Novak-Hofer, I., Amstutz, H. P., Macke, H. R., Schwartzbach, R., Zimmermann, K., Morgenthaler, J., and Schubiger, P. A. (1995) Cellular processing of copper-67-labeled monoclonal antibody chCE7 by human neuroblastoma cells. *Cancer Res. 55*, 46–50.

(41) Press, O. W., Shan, D., Howell-Clark, J., Eary, J., Appelbaum, F. R., Matthews, D., King, D. J., Haines, A., Hamann, P., Hinman, L., Shochat, D., and Bernstein, I. D. (1996) Comparative metabolism and retention of Iodine-125, Yttrium-90, and Indium-111 radioimmunoconjugates by cancer cells. *Cancer Res. 56*, 2123–2129.

BC9901292

Vinyl Sulfone Bifunctional Derivatives of DOTA Allow Sulfhydryl- or Amino-Directed Coupling to Antibodies. Conjugates Retain Immunoreactivity and Have Similar Biodistributions

Lin Li,[†] Shih-Wa Tsai,[†] Anne-Line Anderson,[‡] David A. Keire,[§] Andrew A. Raubitschek,[‡] and John E. Shively*,[†]

Division of Immunology, Beckman Research Institute of the City of Hope, Duarte, California 91010, Department of Radioimmunotherapy, City of Hope Medical Center, Duarte, California 91010, and CURE Digestive Diseases Research Center, UCLA School of Medicine, Los Angeles, California 90095.
Received July 24, 2001; Revised Manuscript Received October 25, 2001

We have synthesized a bifunctional vinyl sulfone–cysteineamido derivative of DOTA (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid) that can be conjugated to the sulfhydryls of mildly reduced recombinant antibody (chimeric anti-CEA antibody cT84.66) at pH 7 or to the amino groups of lysine residues at pH 9. The conjugation is sulfhydryl specific at pH 7 (case 1), and amino specific at pH 9 (case 2) as long as the antibody has no free sulhydryl groups. At a molar ratio of 50 BCA (bifunctional chelating agent) to mAb, the number of chelates conjugated is 0.8 for case 1, and 4.6 for case 2. The resulting conjugates can be radiolabeled with ^{111}In to high specific activity (5 mCi/mg) with high efficiency (>95%) at 43 °C in 60 min. The radiolabeled conjugates retained >95% immunoreactivity and are stable in serum containing 1mM DTPA over 3 d. When the radiolabeled conjugates were injected into nude mice bearing LS174T human colon tumor xenografts, over 40% ID/g accumulated in tumors during the period 24–72h. Tumor-to-blood ratios were 4.5, 3.5, and 2.5 for the sulfhydryl coupled conjugate at 24, 48, and 72 h, respectively, and 2.7, 2.5, and 2.3 for the amino-coupled conjugate at the same time points. For other organs the biodistributions were nearly identical whether the conjugates were attached via sulfhydryl or amino groups. These novel BCAs are easy to synthesize, offer versatile conjugation options, and give equivalent biodistributions that result in high tumor uptake and good tumor-to-blood ratios.

INTRODUCTION

Bifunctional chelating agents (BCAs[1]) are routinely conjugated to recombinant antibodies for radiolabeling with radiometals such as ^{111}In for radioimmunoimaging and ^{90}Y for radioimmunotherapy (*1–4*). The main advantages of this approach is the ability to radiolabel the conjugate just prior to use and the exceptional stability of the chelated metal, preventing the release of the radionuclide from the conjugate. Starting with BCAs derived from EDTA (*5*, *6*), and then DTPA (*7–11*), and more recently DOTA (*12–16*), increasingly more stable radiometal–chelate–antibody conjugates have been crafted. Nonetheless, the approach has certain drawbacks inherent in the biodistribution of the antibody which in the case of intact antibody can be deposited in the liver (*17–21*), and in the case of antibody fragments, in the kidney (*22–25*). High uptake in normal tissues is due to the deposition of free antibody or antibody antigen conjugates in the liver for intact antibody (*21*) and to renal filtering and reabsorption for antibody fragments (*26*). In both cases, the exceptional stability of the radiometal–chelate and the lack of metabolizing enzymes prevents the excretion of these end-products from the lysosomal compartment. While it is assumed that the antibodies are completely degraded in the lysosome, releasing radiometal–chelate, it has only been proven in a few cases (*27–31*). In addition, the site of BCA conjugation to the antibody may play a key role in the fate of the radiolabel in both the tumor and normal organs. Furthermore, it is essential that the BCA be conjugated to the antibody without impairment of immunoreactivity. Since a major goal of the field is to reduce normal tissue uptake without decreasing the tumor uptake, we have investigated new BCA derivatives of DOTA that include the possibility of site-specific conjugation and linker specific cleavage. In previous work we showed that a maleimidocysteineamido derivative of DOTA coupled to sulfhydryl groups of mildly reduced intact antibody underwent pH dependent cleavage at the resulting succinimido groups (*32*), lowering blood levels while preserving high tumor uptake (*33*). However, the results were only a marginal improvement over our use of DOTA-active ester conjugated antibody (*34*, *35*). Oxidation of the thioether linker to a sulfone linker further increased the rate of linker bond cleavage, but the rate of blood clearance was too high, preventing good tumor uptake (*33*). Similar approaches utilizing ester linkers for reducing blood levels have been reported, but also suffer from lower blood levels to the point of reducing

* To whom correspondence should be addressed. e-mail: jshively@coh.org.
[†] Beckman Research Institute of the City of Hope.
[‡] City of Hope Medical Center.
[§] UCLA School of Medicine.

[1] Abbreviations: BCA, bifunctional chelating agent; CEA, carcinoembryonic antigen; DOTA, 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; DTPA, diethylenetriaminepentaacetic acid; HSA, human serum albumin; sulfo-NHS, *N*-hydroxysulfosuccinimide; TCEP, tris(carboxyethyl)phosphine; VSC-DOTA, vinyl sulfone-cysteineamido-DOTA.

10.1021/bc015535b CCC: $22.00 © 2002 American Chemical Society
Published on Web 12/14/2001

tumor uptake (*21*, *36*–*38*). A similar problem is found in using antibody fragments, which clear the blood rapidly, but also give low tumor uptake (*25*). Thus, it is clear that the price of lowering blood levels is the concomitant lowering of tumor uptake, an unacceptable consequence in either radioimmunoimaging or radioimmunotherapy. A possible solution to the problem is the synthesis of BCAs with organ-specific cleavable linkers. Arano and co-workers (*26*, *39*–*41*) have synthesized C-terminal Lys linkers for a radioiodinated hippuric acid–Fab conjugatethat lowered kidney uptake by 50%. Meares and coworkers (*42*–*44*) have synthesized DOTA with cathepsin cleavable linkers for use in lowering liver levels. In the work reported here, we have made new derivatives of DOTA that incorporate a vinyl sulfone-based linker that shows versatility in terms of conjugation to either sulfhydryl or amino groups of antibodies. The BCA is easy to synthesize from readily available chemicals and gives high radiolabeling yields when conjugated to either sulfhydryl or amino groups. The biodistributions in tumor-bearing animals is nearly equivalent, suggesting that the site of conjugation has not affected either the tumor or normal organ uptake. The new BCA may be useful in preventing the impairment of immunoreactivity in cases where coupling through amino groups is problematic.

EXPERIMENTAL PROCEDURES

General. DOTA trisodium salt and the free acid were purchased from Parish Chemical Co. (Orem, UT) and Macrocyclics, Inc. (Richardson, TX), respectively. DTPA was obtained from Fluka (Milwaukee, WI), vinyl sulfone from Aldrich (Milwaukee, WI), Chelex 100 from BioRad (Hercules, CA), and L-cysteine from Sigma (St. Louis, MO). Ultrapure water (18 MΩ-cm) was used to make all buffers that were passed over Chelex 100 and pH adjusted if necessary. $^{111}InCl_3$ was obtained from NEN Life Science products, Inc. (Boston, MA). The anti-CEA chT84.66 has been previously described (*35*, *45*). HPLC and TLC were performed as previously described (*32*). Mass spectra were recorded on a Finnigan LCQ ion trap mass spectrometer using a custom nanospray interface. NMR spectra were recorded on a Varian Unity Plus 500 MHz spectrometer. Proton chemical shifts are reported relative to HOD (4.80 ppm). Antibody concentrations were calculated at 280 nm (A_{280}, 1 mg/mL = 1.42). UV spectrophometry was performed on a Shimadzu UV160 instrument using a 1 cm cell. Melting points were recorded on a Thomas-Hoover uni-melt apparatus and are uncorrected. SDS and IEF gel electrophoresis was performed on a Pharmacia PhastGel apparatus according to the manufacturer's instructions.

Vinyl Sulfone–Cysteineamido–DOTA (VSC-DOTA; 2). Cysteineamido-DOTA (**1**) was synthesized as previously reported (*46*). To a solution of 3.24 mg (6.1 μmol) of **1** in 0.6 mL of 50 mM, pH 7.5 potassium phosphate was added 0.06 mL of 0.1 M DTT (6 mmol) and stirred for 0.5 h at RT under Ar. Vinyl sulfone (6.6 μL (64 μmol) in 0.6 mL of DMF was added with continuous stirring for 5 h at RT. A second aliquot was added and the reaction continued for another 22 h (final vol = 1.2 mL, 5.3 mM **1**, 107 mM vinyl sulfone). The excess vinyl sulfone was extracted with ethyl acetate (2 mL, ×3). The aqueous layer was adjusted to pH 8.4, applied to a BioRad Ag1-X4 column (HCO_2^- form, 1 × 10 cm), washed with 30 mL of water, and eluted stepwise with 0.05–2 M HCO_2H (5 mL per step). The product (**2**) was found in fractions 8–13 by ESI/MS. The product was repeatedly lyophilyzed from water to remove excess HCO_2H (yield 3.4 mg). The product had the expected protonated mass by ESI/MS (626.71, expected; 626.73, observed), gave a single spot on TLC, and had a melting point of 140–142 °C. Proton NMR (H_2O): δ 2.92 (m, 2H, $SCH_2CH_2SO_2$), δ 3.10 (m, 2H, $SCH_2CH_2SO_2$), δ 3.10–3.45 [br, 16H, $(CH_2NCH_2) \times 4$], δ 3.53 (dd, 2H, CH_2SO_2), δ 3.45, 3.70, 3.84 (s, 6H, NCH_2CO_2H), δ 4.39 (m, 1H, $NHCHCO_2H$), δ 6.42 (dd, 2H, SO_2CHCH_2), δ 6.88 (dd, 1H, SO_2CHCH_2), δ 8.20 (d, 1H, CONH–CH).

Preparation of Amino-Conjnugated BCA Antibody (VSC-DOTA-NH-chT84.66). chT84.66 (FW= 150000, 1.0 mg, 6 nmol, at 10 mg/mL, 60 μM) was dialyzed vs 20 mM, pH 7.0 sodium phosphate buffer (three changes, × 1 L, 4 °C) and adjusted to pH 9.0 with 200 mM, pH 10 sodium phosphate (final volume, 0.1 mL). VSC-DOTA (0.1 mL of **2**, FW= 625, 0.7 mg, 1.12 μmol at 1.88 mg/mL, 3.1 mM in 50 mM, pH 9.0 sodium phosphate) was added and the solution rotated at 10 rpm for 4 h at 25 °C. The conjugate was dialyzed vs 250 mM NH_4 acetate (three changes, × 1 L, 4 °C).

Preparation of Sulfhydryl-Conjnugated BCA Antibody (VSC-DOTA-SH-chT84.66). chT84.66 was dialyzed as above and reduced with TCEP (20 μL of 10 mM in 50 mM sodium phosphate, pH 7.0) under argon for 2.5 h at 25 °C. TCEP was removed on a spin column (1 mL of Sephadex G25–50, 1200 rpm, 2 min) under argon. To 1.0 mg (60 nmol) of reduced chT84.66 in 0.1 mL of 50 mM sodium phosphate, pH 7, was added 0.1 mL of 3 mM (300 nmol) of VSC-DOTA (**2**) in the same buffer. The solution was rotated under argon overnight at 25 °C and dialyzed vs NH_4 acetate as described above. In parallel, 1 mg of TCEP reduced chT84.66 in 0.15 mL was reacted with 0.1 mL of 3 mM MC-DOTA and run as a control.

Radiolabeling of VSC-DOTA-chT84.66. The conjugates were radiolabeled with $^{111}InCl_3$ as previously described (*47*). For the animal studies, the conjugates (0.3 mL of 1 mg/mL in 250 mM NH_4 acetate, pH 5.7) were incubated with 0.3 mCi of ^{111}In (0.15 mL of 0.04 M HCl plus 0.03 mL of 250 mM NH_4 acetate, pH 5.7) for 60 min at 43 °C. Excess ^{111}In was chelated with 1 mM DTPA and the radiolabeled product purified on a TosoHaas TSK–Gel G2000SW column (0.75 × 30 cm in saline, 0.5 mL/min, 0.5 mL fractions). The protein peak was mixed with 1 drop of 25% (w/v) of HSA. In other test labelings, 1.0 mCi of ^{111}In was added to 0.2 mg of conjugate and the specific activity determined by ITLC (kit 151–770, Biodex, Medical, New York). Determination of the number of chelates per conjugate and the immunoreactivity was performed as previously described (*5*).

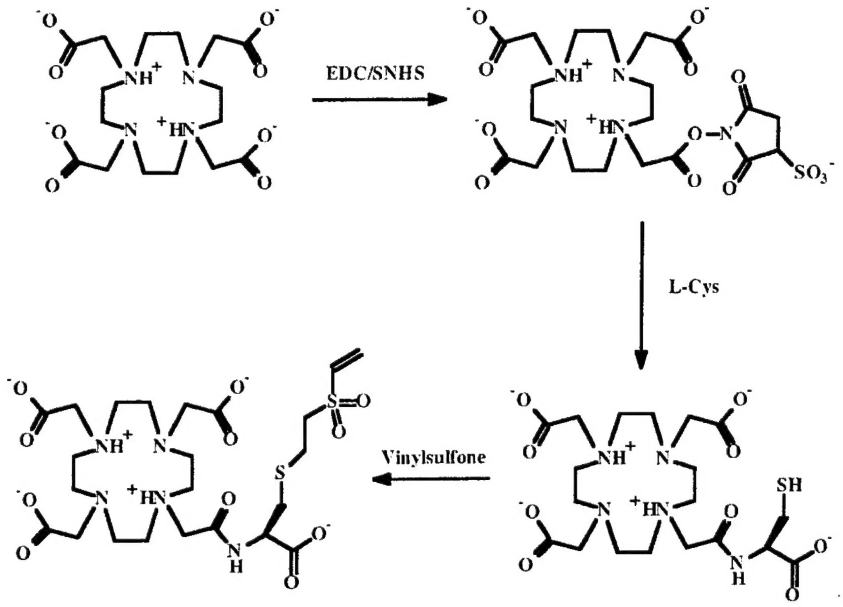
Stability Studies. Aliquots (0.5 mL) of the radiolabeled conjugates in 1% HSA were mixed with 0.05 mL of 10 mM DTPA, stored at 37 °C and injected at various time points onto a Pharmacia Superose 12HR column (as above) to determine the amount of radiolabel in the conjugate and DTPA.

Animal Biodistributions. Groups of five nude mice (Charles River) bearing LS174T human colon tumor xenografts (ave size = 1.5 mg ± 0.5 at t = 0 h) were injected with 2 μCi (2 μg) of radiolabeled conjugate via tail vein. Animals were sacrificed at 0, 12, 24, 48, 72, and 96 h after injection. Major organs, tumor, and blood were weighed, radioactivity was measured, and the results were expressed as mean %ID/g ± SD.

RESULTS

Synthesis and Antibody Conjugation. The synthesis of VSC-DOTA (**2**) is shown in Scheme 1. The product gave the expected proton NMR spectrum and protonated

Scheme 1



mass by ESI/MS and was free of DOTA and the bis-derivative. The reactivity of **2** to free L-cysteine was confirmed by ESI/MS (data not shown). At pH 7, only the mono-derivative was formed, suggesting that the thiol and not the α-amino group of L-cysteine was reactive under these conditions. When VSC-DOTA was incubated for 1−7 d in pH 7 PBS and analyzed by RP-HPLC, there was no evidence of chemical instability (data not shown).

Since VSC-DOTA was expected to react with the ϵ-amino group on the lysines of proteins at pH 9−10, we investigated the reaction of VSC-DOTA with antibody over the pH range of 7−9. The degree of conjugation was determined by IEF gel electrophoresis, wherein conjugation of the acidic BCA to antibody results in a dramatic shift of the antibody to a more acidic pI. The results of the conjugation reaction at pH 7, 8, and 9 shown in Figure 1A, lanes 3−5, demonstrate that little or no reaction occurs at pH 7, while substantial amounts of conjugate are formed at pH 9. The results at pH 8 are intermediate, with <20% product formed. The results at 37 °C, pH 9 demonstrate the expected effect of temperature on the extent of reaction (Figure 1A, lane 6). The products show no change in mobility when run on SDS gel electrophoresis under nonreducing conditions (data not shown). On the basis of these results, we reasoned that sulfhydryl specific conjugation could be performed at pH 7 with little or no conjugation at amino groups, while amino group specific conjugation could be performed at pH 9, as long as no free sulfhydryl groups are present. Since the cysteine residues in most antibodies are engaged in disulfide bonds, this criterion is easily met. In addition, under mild reducing conditions (no denaturing agents), only the hinge disulfides of antibodies are reduced, allowing site-specific conjugation at just these sulfhydryl groups (*48*, *49*). The conjugation of VSC-DOTA to mildly reduced chT84.66 at pH 7 shown in Figure 1B, lane 4, demonstrates that >90% of the antibody is conjugated under these conditions. It should be noted that either DTT or TCEP were successfully used as reducing agents. We prefer TCEP because a lower molar excess is required and since removal of the reducing agent by spin columns is not quantitative, better conjugation yields are routinely obtained.

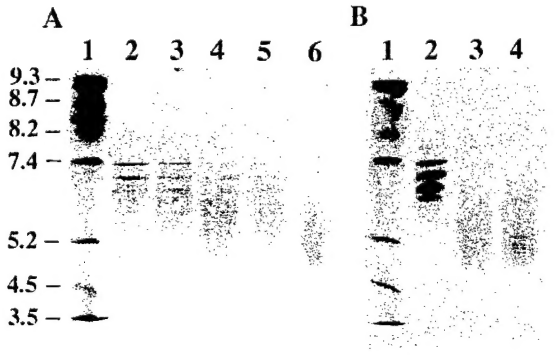


Figure 1. Analysis of VSC-DOTA-mAb conjugates by IEF gel electrophoresis. A: Lane 1: IEF standards; Lane 2: chT84.66 control; Lane 3: 30 μM chT84.66, 1.5 mM VSC-DOTA, pH 7.0, RT, 4h; Lane 4: 30 μM chT84.66, 1.5 mM VSC-DOTA, pH 8.0, RT, 4 h; Lane 5: 30 μM chT84.66, 1.5 mM VSC-DOTA, pH 9.0, RT, 4 h; Lane 6: 30 μM chT84.66, 1.5 mM VSC-DOTA, pH 9.0, 37 °C, 4 h. B: Lane 1: IEF standards; Lane 2: chT84.66 control; Lane 3: 30 μM chT84.66 reduced with 0.5 mM TCEP, the TCEP removed by spin column, and reacted with 1.5 mM MC-DOTA, pH 7.0, RT, 4 h; Lane 4: 30 μM chT84.66, reduced with 0.5 mM TCEP, the TCEP removed by spin column, and reacted with 1.5 mM VSC-DOTA, pH 7.0, RT, 4 h.

The number of chelates per antibody as determined by trace labeling with ^{111}In in a standard solution of $InCl_3$ is shown in Table 1. At pH 7 and with a 50:1 molar ratio of VSC-DOTA to mAb, mildly reduced antibody gave 0.8 chelates/mAb out of a theoretical of 8 for a human IgG1 hinge. At pH 9 with the same molar ratio of VSC-DOTA to mAb, unreduced mAb gave 4.6 chelates/mAb. Radiolabeling of these two conjugates with $^{111}InCl_3$ gave similar percent radiolabeling efficiencies, specific activities, and

Table 1. Radiometal Labeling and Immunoreactivity of VSC-DOTA-mAb[a]

sample	chelates/ mAb	labeling ratio (mCi/mg)	% efficiency	% immuno-reactivity
SH-mAb	0.8	1.0−5.0	>98	>98
NH-mAb	4.5	1.0−5.0	>98	>98

[a] VSC-DOTA was coupled to either SH or NH groups in the mAb as described in the text. The number of chelates per mAb was determined by trace labeling with ^{111}In in a standard solution of $InCl_3$. The labeling ratio was performed with 0.3 mCi of ^{111}In and 0.3 mg of conjugate in 0.3 mL of 0.25 mM ammonium acetate (pH 5.7), or 1.0 mCi of ^{111}In and 0.2 mg of conjugate in 0.2 mL of ammonium actetate (pH 5.7). Immunoreactivity was performed as described in Methods.

Table 2. Biodistribution of ^{111}In-Labeled VSC-DOTA-SH mAb in Nude Mice Bearing LS174T Xenografts[a]

	0 h	12 h	24 h	48 h	72 h	96 h
blood	47.97	23.83	15.00	7.33	3.98	2.49
	(2.58)	(2.43)	(2.07)	(3.78)	(2.51)	(3.02)
liver	7.30	6.65	10.15	14.91	19.60	18.83
	(0.25)	(1.94)	(1.90)	(6.27)	(7.19)	(3.32)
spleen	5.41	5.18	5.93	7.76	6.41	7.69
	(1.22)	(0.57)	(0.95)	(2.54)	(2.25)	(2.27)
kidney	6.36	5.62	4.36	3.46	2.82	2.63
	(1.43)	(0.46)	(0.56)	(1.08)	(0.46)	(0.43)
lung	14.01	8.59	5.50	2.99	1.87	0.90
	(3.27)	(1.26)	(0.72)	(1.36)	(0.88)	(0.59)
tumor	1.59	37.08	43.61	42.01	40.43	39.57
	(0.47)	(4.09)	(4.91)	(12.73)	(6.96)	(18.97)
carcass	1.57	2.58	2.15	1.47	1.05	0.96
	(0.15)	(0.10)	(0.29)	(0.48)	(0.26)	90.51)

[a] Five animals per group were injected with 4 μCi (4 μg) of VSC-DOTA coupled to chT84.66 via the hinge SH groups and radiolabeled with ^{111}In. The results are reported as mean %ID/g (±SD).

Table 3. Biodistribution of ^{111}In-Labeled VSC-DOTA-NH mAb in Nude Mice Bearing LS174T Xenografts[a]

	0 h	12 h	24 h	48 h	73 h	96 h
blood	47.61	17.98	17.50	8.98	5.84	3.08
	(2.97)	(6.29)	(6.74)	(3.18)	(4.14)	(4.50)
liver	9.16	12.38	13.93	16.27	19.64	18.42
	(0.97)	(3.77)	(5.75)	(4.25)	(5.36)	(4.79)
spleen	7.25	6.35	8.09	7.77	10.07	9.06
	(0.68)	(0.73)	(2.34)	(2.00)	(2.08)	(2.09)
kidney	7.29	4.24	5.03	3.44	2.60	2.15
	(1.36)	(1.14)	(1.77)	(0.87)	(0.79)	(0.76)
lung	13.07	5.63	6.58	3.44	2.58	1.45
	(1.18)	(1.79)	(2.58)	(1.30)	(1.57)	(1.63)
tumor	1.26	21.66	38.54	39.27	45.76	33.85
	(0.40)	(5.02)	(13.60)	(10.19)	(18.25)	(14.52)
carcass	1.76	2.20	2.64	1.72	1.43	0.89

[a] Five animals per group were injected with 4 μCi (3.4 μg) of VSC-DOTA coupled to chT84.66 via NH groups and radiolabeled with ^{111}In. The results are reported as mean %ID/g (± SD).

immunoreactivities (Table 1). When the radiolabeled conjugates were incubated in 1% HSA and 1 mM DTPA for 1−7d, negligible loss of radioactivity was seen to DTPA (Table 1). In addition, their immunoreactivities were >95% when radiolabeled, bound to excess CEA, and analyzed by gel filtration chromatography. Since their immunoreactivities were similar, we used these conjugates for a direct comparison in animal biodistribution studies.

Comparative Animal Biodistributions of [^{111}In]-VSC-DOTA-SH-chT84.66 and [^{111}In]VSC-DOTA-NH-chT84.66. The two ^{111}In-radiolabeled conjugates were injected into nude mice bearing human colon tumor xenografts and their biodistributions compared (Tables 2 and 3). The overall biodistributions are quite similar, resulting in maximal and sustained tumor uptake of 35−45% ID/g from 24 to 96 h. During the same time period the blood activity drops from about 45% ID/g to 2−3% ID/g. A comparison of tumor to blood curves over the time course suggests that the SH conjugate may be superior to the NH conjugate. Paradoxically, this difference may be due to a slightly slower blood clearance curve for the SH conjugate, resulting in higher uptake. A close analysis of the normal organs reveals nearly identical uptake levels and time courses for the spleen, kidney, and lung, but there are some interesting differences in the liver. The SH conjugate shows lower liver uptake at the earlier time points, suggesting that the NH conjugate accumulates more rapidly in the liver. This result would also account for their observed differences in blood clearance curves.

DISCUSSION

In a search for site-specific BCA derivatives of DOTA we first synthesized a bis-maleimidohexane derivative of cysteineamido-DOTA (*32*). Maleimide is a commonly used reagent that undergoes sulfhydryl specific Michael additions that are thought to be chemically stable (*50*). Although this BCA allowed site-specific conjugation to the sulfhydryl groups of mildly reduced mAb, it proved to be chemically labile, undergoing pH-dependent hydrolysis at its resulting succinimidyl bonds (*32*). Surprisingly, this phenomenon did not prevent good tumor uptake in animal biodistribution studies (*33*). As a result, we proposed that further increasing the chemical lability of the linker may be beneficial, since this would result in lower blood levels. Indeed, oxidation of the thioether bond in the linker to a sulfone increased the chemical lability of the BCA (*32*), but the BCA decomposed too rapidly to allow good tumor uptake. On the basis of these studies, we decided to investigate other Michael addition linkers as candidates for site-specific conjugation to mAbs. We chose vinyl sulfone for its well-documented reaction with sufhydryl groups (*51*). However, we realized from the outset that vinyl sulfone can also react with amino groups (*52*, *53*), and thus we were interested in seeing if this reaction could be controlled by manipulation of the reaction pH.

Vinyl sulfone is a viscous liquid with poor solubility in water, but when dissolved in DMF or acetonitrile reacts well with Michael acceptors in aqueous medium. In the synthesis of the title compound (**2**), we demonstrated a facile reaction between vinyl sulfone and cysteineamido-DOTA. Unlike the maleimido derivatives, the vinyl sulfone derivative of cysteineamido-DOTA is stable in PBS at pH 7. When **2** was reacted with chT84.66 at pH 7, no conjugate was formed unless the mAb was mildly reduced prior to reaction. In addition, we showed that the mAb conjugated with **2** at pH 9 to a greater extent (no. chelates/mAb) as it did to reduced mAb at pH 7 using the same molar ratio of **2** to mAb. Thus, we demonstrated that this BCA can be conjugated to the same mAb via different chemical linkages using pH and reducing agents to convey linkage specificity.

Since the biodistributions of radiolabeled mAbs is highly dependent on the mode of attachment of the radiolabel, it is always interesting to compare BCAs conjugated to the same mAb via different chemical linkages. In most cases, it is difficult to perform this type of comparative study. However, in this study, we have an almost ideal model. In one case, the same BCA is conjugated to sulfhydryl groups, and in the other case, amino groups, but the resulting BCA/mAb ratios are different. Even so, the resulting biodistributions are quite similar. When we compare these results to our previous studies in which DOTA was conjugated to chT84.66 by an active ester derivative of DOTA (*34*, *35*), we find that the uptake curves are similar for both tumor and normal tissues. This suggests that linkage of DOTA based BCAs to mAbs via amides, amine, or sulfhydryl bonds results in similar tissue, including tumor, uptakes, and metabolism. Thus, if one wants to manipulate normal or tumor uptake and metabolism of DOTA-based BCAs, it is necessary to explore other modes of chemical linkage or to insert linkers which have built-in chemical or biological lability. Examples of such linkers have been described by Meares (*21*), Arano (*54*), and ourselves (*32*).

When we examined the comparative biodistributions of the SH and NH BCA-chT84.66 conjugates in more detail, we noticed some intriguing trends. They include

the higher blood levels, higher tumor levels, and lower liver levels for the BCA chT84.66 SH conjugate at early time points 0−24 h. These three "organs" are interdependent in their biodistributions as follows: high tumor uptake is dependent on maintaining high blood levels, especially early in a biodistribution. If the blood is cleared by any mechanism, including rapid liver uptake, then blood and tumor levels will fall together. Thus, the lower liver uptake seen at early time points for the SH conjugate leads to higher blood levels and ultimately to higher tumor uptake which is sustained at later time points. While the biological reasons for these differences are unknown at this time, we can speculate that this mode of conjugation will lead to incremental improvements in the human, where most biodistributions are predicted from the animal model.

In summary, we have prepared a new BCA that allows site-specific conjugation to most mAbs either via sulfhydryl or amino groups by manipulating pH and use of reducing agents. The ^{111}In-radiolabeled versions of these conjugates give more or less equivalent animal biodistributions to previously described ^{111}In-radiolabeled BCA-mAbs, including are own version using the active ester of DOTA (*34*, *35*). This approach should have utility, allowing the investigator to chose one type of chemistry over the other depending on the restrictions implied by a given antibody. Finally, the improvement in tumor to blood and tumor-to-liver ratios seen for the SH-specific conjugate may translate into improved performance in the clinic.

ACKNOWLEDGMENT

The authors thank Stefanie Wright for the animal studies. This research was funded by NCI grant CA43904 (A.A.R. and J.E.S.) and the Department of the Army Grant DAMD 17-98-1-8031 (J.E.S.).

LITERATURE CITED

(1) Anderson, C. J., and Welch, M. J. (1999) Radiometal-labeled agents (non-technetium) for diagnostic imaging. *Chem. Rev. 99*, 2219−2234.

(2) Gansow, O. A. (1991) Newer Approaches to the Radiolabeling of Monoclonal Antibodies by Use of Metal Chelates. *Int. J. Radiat. Appl. Instrum. Part B Nucl. Med. Biol. 18*, 369−381.

(3) Meares, C. F. (1986) Chelating Agents for the Binding of Metal Ions to Antibodies. *Int. J. Radiat. Appl. Instrum. Part B Nucl. Med. Biol. 13*, 311−318.

(4) Liu, S., and Edwards, D. S. (2001) Bifunctional chelators for therapeutic lanthanide radiopharmaceuticals. *Bioconjugate Chem. 12*, 7−34.

(5) Meares, C. F., McCall, M. J., Reardan, D. T., Goodwin, D. A., Diamanti, C. I., and McTigue, M. (1984) Conjugation of Antibodies with Bifunctional Chelating Agents: Isothiocyanate and Bromoacetamide Reagents, Methods of Analysis, and Subsequent Addition of Metal Ions. *Anal. Biochem. 142*, 68−78.

(6) Brechbiel, M. W., Gansow, O. A., Atcher, R. W., Schlom, J., Esteban, J., Simpson, D. E., and Colcher, D. (1986) Synthesis of 1-(*p*-Isothiocyanatobenzyl) Derivatives of DTPA and EDTA. Antibody Labeling and Tumor-Imaging Studies. *Inorg. Chem. 25*, 2772−2781.

(7) Brechbiel, M. W., and Gransow, O. A. (1991) Backbone-substituted DTPA ligands for ^{90}Y radioimmunotherapy. *Bioconjugate Chem. 2*, 187−194.

(8) Cummins, C. H., Rutter, E. W., and Fordyce, W. A. (1991) A convenient synthesis of bifunctional chelating agents based on diethylenetriaminepentaacetic acid and their coordination chemistry with yttrium (III). *Bioconjugate Chem. 2*, 180−186.

(9) Hnatowich, D. J., Virzi, F., and Doherty, P. W. (1985) DTPA-Coupled Antibodies Labeled with Yttrium-90. *J. Nucl. Med. 26*, 503−509.

(10) Westerberg, D. A., Carney, P. L., Rogers, P. E., Kline, S. J., and Johnson, D. K. (1989) Synthesis of Novel Bifunctional Chelators and Their Use in Preparing Monoclonal Antibody Conjugates for Tumor Targeting. *J. Med. Chem. 32*, 236−243.

(11) Corson, D. T., and Meares, C. F. (2000) Efficient multigram synthesis of the bifunctional chelating agent (S)-1-p-isothiocyanatobenzyl-diethylenetetraaminepentaacetic acid. *Bioconjugate Chem. 11*, 292−299.

(12) Cox, J. P. L., Jankowski, K. J., Kataky, R., Parker, D., Beeley, N. R. A., Boyce, B. A., Eaton, M. A. W., Millar, K., Millican, A. T., Harrison, A., and Walker, C. (1989) Synthesis of a Kinetically Stable Yttrium-90 Labeled Macrocycle-Antibody Conjugate. *J. Chem. Soc., Chem. Commun.* 797−798.

(13) Deshpande, S. V., DeNardo, S. J., Kukis, D. L., Moi, M. K., McCall, M. J., DeNardo, G. L., and Meares, C. F. (1990) Yttrium-90-labeled monoclonal antibody for therapy: labeling by a new macrocyclic bifunctional chelating agent. *J. Nucl. Med. 31*, 473−479.

(14) Meares, C. F., Moi, M. K., Diril, H., Kukis, D. L., McCall, M. J., Deshpande, S. V., DeNardo, S. J., Snook, D., and Epenetos, A. A. (1990) Macrocyclic chelates of radiometals for diagnosis and therapy. *Br. J. Cancer 62*, Suppl. X, 21−26.

(15) Smith-Jones, P. M., Fridrich, R., Kaden, T. A., Novak-Hofer, I., Siebold, K., Tschudin, D., and Maecke, H. R. (1991) Antibody Labeling with Copper-67 Using the Bifunctional Macrocycle 4-[(1,4,8,11-Tetraazacyclotetradec-1-yl)methyl]-benzoic Acid. *Bioconjugate Chem. 2*, 415−421.

(16) Kline, S. J., Betebenner, D. A., and Johnson, D. K. (1991) Carboxymethyl-Substituted Bifunctional Chelators: Preparation of Aryl Isothiocyanate Derivatives of 3-(Carboxymethyl)-3-azapentanedioic Acid, 3,12-Bis(carboxymethyl)-6,9-dioxa-3,12-diazatetradecanedioic Acid, and 1,4,7,10-Tetraazacyclododecane-*N*,*N*′,*N*″,*N*‴-tetraacetic Acid for Use as Protein Labels. *Bioconjugate Chem. 2*, 26−31.

(17) Halpern, S. E., Hagan, P. L., Garver, P. R., Koziol, J. A., Chen, A. W. N., Frincke, J. M., Bartholomew, R. M., David, G. S., and Adams, T. H. (1983) Stability, Characterization, and Kinetics of ^{111}In-labeled Monoclonal Antitumor Antibodies in Normal Animals and Nude Mouse-Human Tumor Models. *Cancer Res. 43*, 5347−5355.

(18) Jakowatz, J. G., Beatty, B. G., Vlahos, W. G., Porudominsky, D., Philben, V. J., Williams, L. E., Paxton, R. J., Shively, J. E., and Beatty, J. D. (1985) High-Specific-Activity ^{111}In-labeled Anticarcinoembryonic Antigen Monoclonal Antibody: Biodistribution and Imaging in Nude Mice Bearing Human Colon Cancer Xenografts. *Cancer Res. 45*, 5700−5706.

(19) Larson, S. M., and Carrasquillo, J. A. (1988) Advatages of radioiodine over radioindium labeled monoclonal antibodies for imaging solid tumors. *Nucl. Med. Biol. 15*, 231−233.

(20) Wong, J. Y. C., Thomas, G. E., Yamauchi, D. M., Williams, L. E., Odom-Maryon, T. L., E. J. M., Neumaier, M., Wu, A. M., Primus, F. J., Shively, J. E., and Raubitschek, A. A. (1997) Clinical evaluation of an ^{111}In labeled anti-CEA chimeric monoclonal antibody in patients with metastatic CEA producing malignancies. *J. Nucl. Med. 38*, 1951−1959.

(21) Meares, C. F., McCall, M. J., Deshpande, S. V., DeNardo, S. J., and Goodwin, D. A. (1988) Chelate radiochemistry: cleavable linkers lead to altered levels of radioactivity in the liver. *Int. J. Cancer (suppl.) 2*, 99−102.

(22) Bujis, W. C. A. M., Massuger, L. F. A. G., Claessens, R. A. M. J., Kenemans, P., and Corstens, F. H. M. (1992) Dosimetric evaluation of immunoscintigraphy using indium-11-labeled monoclonal antibody fragments in patients with ovarian cancer. *J. Nucl. Med. 33*, 1113−1120.

(23) Govindan, S. V., Shih, L. B., Goldenberg, D. M., Sharkey, R. M., Karacay, H., Donnelly, J. E., Losman, M. J., Hansen, H. J., and Griffiths, G. L. (1998) 90-Yttrium-labeled complementarity-determining-region grafted monoclonal antibodies for radioimmunotherpay: radiolabeling and animal biodistribution studies. *Bioconjugate Chem. 9*, 773−782.

(24) Yemul, S., Leon, J. A., Pozniakoff, T., Esser, P. D., and Estabrook, A. (1993) Radioimmunoimaging of human breast carcinoma xenografts in nude mouse model with ^{111}In labeled new monoclonal antibody EBA-1 and F(ab')2 fragments. *Nucl. Med. Biol. 20*, 325−335.

(25) Yokota, T., Milenic, D. E., Whitlow, M., and Schlom, J. (1992) Rapid tumor penetration of a single-chain Fv and comparison with other immunoglobulin forms. *Cancer Res. 52*, 3402−3408.

(26) Arano, Y. (1998) Strategies to reduce renal radioactivity levels of antibody fragments. *Quart. J. Nucl. Med. 42*, 262−270.

(27) Duncan, J. R., and Welch, M. J. (1993) Intracellular metabolism of indium-111 DTPA labeled receptor targeted proteins. *J. Nucl. Med. 34*, 1728−1738.

(28) Duncan, J. R., Stephenson, M. T., Wu, H. P., and Anderson, C. J. (1997) Indium-111-diethylenetriamine pentaacetic acid octreotide is delivered in vivo to pancreatic, tumor cell, renal, and hepatocyte lysosomes. *Cancer Res. 57*, 659−671.

(29) Franano, F. N., Edwards, W. B., Welch, M. J., and Duncan, J. R. (1994) Metabolism of receptor targeted ^{111}In-DTPA-glycoporteins: identification of ^{111}In-DTPA-epsilon-lysine as the primary metabolic and excretory product. *Nucl. Med. Biol. 21*, 1023−1034.

(30) Novak-Hofer, I., Amstutz, H. P., Macke, H. R., Schwartzbach, R., Zimmermann, K., Morgenthaler, J.-J., and Schubiger, P. A. (1995) Cellular processing of copper-67 labeled monoclonal antibody chCE7 by human neuroblastoma cells. *Cancer Res. 55*, 46−50.

(31) Tsai, S. W., Li, L., Williams, L. E., Anderson, A.-L., Raubitschek, A. A., and Shively, J. E. (2001) Metabolism and renal clearance of ^{111}In-labeled DOTA conjugated antibody fragments. *Bioconjugate Chem. 12*, 264−270.

(32) Lewis, M. R., and Shively, J. E. (1998) Maleimidocysteineamido-DOTA derivatives. New reagents for radiometal chelate conjugation to antibody sulfhydryl groups undergo pH-dependent clevage reactions. *Bioconjugate Chem. 9*, 72−86.

(33) Williams, L. E., Lewis, M. R., Bebb, G. G., Clarke, K. G., Odom-Maryon, T. L., Shively, J. E., and Raubitschek, A. A. (1998) Biodistribution of ^{111}In and ^{90}Y labeled DOTA and maleimidocysteineamido-DOTA conjugated to chimeric anti-carcinoembryonic antigen antibody in xenograft bearing nude mice: comparison of stable and chemically labile linker systems. *Bioconjugate Chem. 9*, 87−93.

(34) Lewis, M. R., Raubitschek, A. A., and Shively, J. E. (1994) A facile, water-soluble method for modification of proteins with DOTA. Use of elevated temperature and optimized pH to achieve high specific activity and high chelate stability in radiolabeled immunoconjugates. *Bioconjugate Chem. 5*, 565−576.

(35) Williams, L. E., Primus, F. J., Wong, J. Y. C., Wu, A. M., Odom-Maryon, T. L., Johnson, D. K., Hefta, L. J. F., Shively, J. E., and Raubitschek, A. A. (1996) Biodistribution of an ^{111}In or ^{90}Y labeled chimeric anti-CEA monoclonal antibody (cT84.66) following its large scale production in a bioreactor. *Tumor Targeting 2*, 116−124.

(36) Haseman, M. K., Goodwin, D. A., Meares, C. F., Kaminski, M. S., Wensel, T. G., McCall, M. J., and Levy, R. (1986) Metabolizable 111-In-chelate conjugated anti-iodiotype monoclonal antibody for radioimmunodetection of lymphoma in mice. *Eur. J. Nucl. Med. 12*, 455−460.

(37) Paik, C. H., Yokoyama, K., Reynolds, J. C., Quadri, S. M., Min, C. Y., Shin, Y., Maloney, P. J., Larson, S. M., and Reba, R. C. (1989) Reduction of background activities by introduction of a diester linkage between antibody and a chelate in radioimmunodetection of tumor. *J. Nucl. Med. 30*, 1693−1701.

(38) Deshpande, S. V., DeNardo, S. J., Meares, C. F., McCall, M. J., Adams, G. P., and DeNardo, G. L. (1989) Effect of different linkage between chelates and monoclonal antibodies on levels of radioactivity in the liver. *Nucl. Med. Biol. 16*, 587−597.

(39) Arano, Y., Mukai, T., Uezono, T., Wakisaka, K., Motonari, H., Akizawa, H., Taoka, Y., and Yokoyama, A. (1994) A biological method to evaluate bifunctional chelating agents to label antibodies with metallic radionuclides. *J. Nucl. Med. 35*, 890−898.

(40) Arano, Y., Wakisaka, K., Ohmono, Y., Uezono, T., Akizawa, H., Nakayama, M., Sakahara, H., Tanaka, C., Konishi, J., and Yokoyama, A. (1996) Assessment of radiochemical design of antibodies using an ester bond as the metabolizable linkage: evaluation of maleimidoethyl 3-(tri-*n*-butylstannyl)-hippurate as a radioiodination reagent of antibodies for diagnostic and therapeutic applications. *Bioconjugate Chem. 7*, 628−637.

(41) Fujioka, Y., Arano, Y., Ono, M., Uehara, T., Ogawa, K., Namba, S., Saga, T., Nakamoto, Y., Mukai, T., Konishi, J., and Saji, H. (2001) Renal metabolism of 3′-iodohippuryl Nϵ-maleoyl-L-lysine (HML)-conjugated Fab fragments. *Bioconjugate Chem. 12*, 178−185.

(42) Li, M., and Meares, C. F. (1993) Synthesis, metal chelate stability studies, and enzyme digestion of a peptide-linked DOTA derivative and its corresponding radiolabeled immunoconjugates. *Bioconjugate Chem. 4*, 275−283.

(43) Peterson, J. J., Pak, R. H., and Meares, C. F. (1999) Total solid-phase synthesis of 1,4,7,10-tetraazacyclododecane-N,N′,N″,N‴-tertraacetic acid functionalized peptides for radioimmunotherpay. *Bioconjugate Chem. 10*, 316−320.

(44) Peterson, J. J., and Meares, C. F. (1999) Enzymatic cleavage of peptide linked radiolabels from immunoconjugates. *Bioconjugate Chem. 10*, 553−557.

(45) Neumaier, M., Shively, L., Chen, F.-S., Gaida, F.-J., Ilgen, C., Paxton, R. J., Shively, J. E., and Riggs, A. D. (1990) Cloning of the Genes for T84.66, an Antibody That Has a High Specificity and Affinity for Carcinoembryonic Antigen, and Expression of Chimeric Human/Mouse T84.66 Genes in Myeloma and Chinese Hamster Ovary Cells. *Cancer Res. 50*, 2128−2134.

(46) Lewis, M. R., and Shively, J. E. (1998) Maleimidocysteineamido-DOTA Derivatives: New Reagents for Radiometal Chelate Conjugation to Antibody Hinge Region Sulfhydryl Groups Undergo pH-Dependent Cleavage Reactions. *Bioconjugate Chem. 9*, 72−86.

(47) Williams, L. E., Lewis, M. R., Bebb, G. G., Clarke, K. G., Odom-Maryon, T. L., Shively, J. E., and Raubitschek, A. A. (1998) Biodistribution of ^{111}In- and ^{90}Y-Labeled DOTA and Maleimidocysteineamido-DOTA Conjugated to Chimeric Anti-Carcinoembryonic Antigen Antibody in Xenograft-Bearing Nude Mice: Comparison of Stable and Chemically Labile Linker Systems. *Bioconjugate Chem. 9*.

(48) Gunewardena, P., and Cook, K. B. (1966) 1,4-Dithiothreitol as a Reagent for Reductive Degradation of Human γG Globulin. *Biochem. J. 99*, 8P.

(49) Willner, D., Trail, P. A., Hofstead, S. J., King, H. D., Lasch, S. J., Braslawsky, G. R., Greenfield, R. S., Kaneko, T., and Firestone, R. A. (1993) (6-Maleimidocaproyl)hydrazone of Doxorubicin- -A New Derivative for the Preparation of Immunoconjugates of Doxorubicin. *Bioconjugate Chem. 4*, 521−527.

(50) Gregory, J. D. (1955) The Stability of *N*-Ethylmaleimide and its Reaction with Sulfhydryl Groups. *J. Am. Chem. Soc. 77*, 3922−3923.

(51) Houen, G., and Jensen, O. M. (1995) Conjugation to preactivated proteins using divinyl sulfone and iodoacetic acid. *J. Immunol. Methods 181*, 187−200.

(52) Friedman, M., and Finley, J. W. (1975) Reactions of proteins with ethyl vinyl sulfone. *Int. J. Pept. Protein Res. 7*, 481−486.

(53) Masri, M. S., and Friedman, M. (1988) Protein reactions with methyl and ethyl vinyl sulfones. *J. Protein Res. 7*, 49−54.

(54) Arano, Y., Fujioka, Y., Akizawa, H., Ono, M., Uehara, T., Wakisaka, K., Nakayama, M., Sakahara, H. K. J., and Saji, H. (1999) Chemical design of radiolabeled antibody fragments for low renal radioactivity levels. *Cancer Res. 59*, 128−134.

BC015535B